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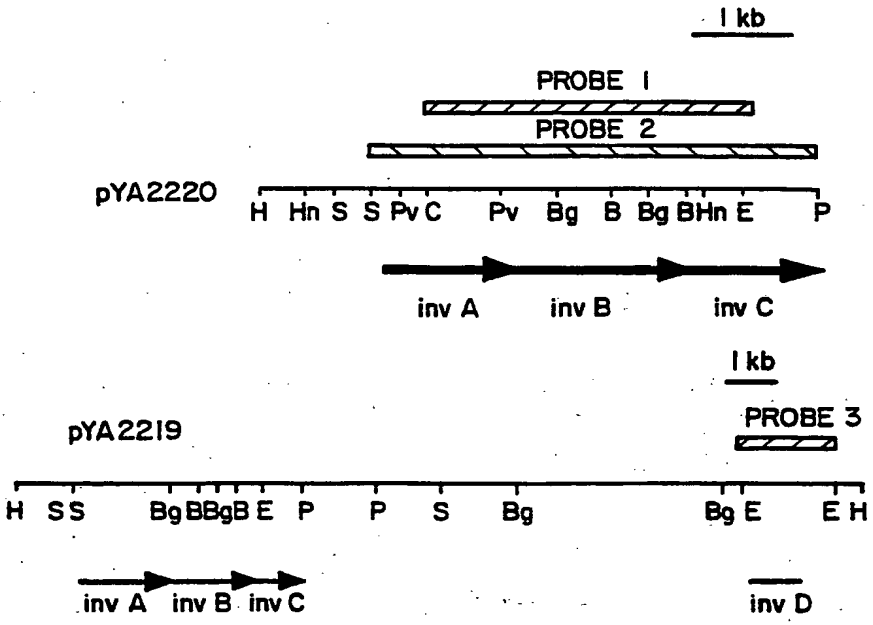

(57) Abstract

Polynucleotide probes and primers derived from the *Salmonella typhimurium inv* genes are described. These polynucleotides can be used as universal probes and primers to detect the presence or absence of *Salmonella* nucleotide sequences in a biological sample.

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POLYNUCLEOTIDE PROBES FOR SALMONELLA

Technical Field

The present invention relates to methods and materials for identifying microorganisms. More specifically, the instant invention pertains to methods for detecting Salmonella using polynucleotide probes and primers derived from the Salmonella typhimurium invA, B, C, and D genes.

Background of the Invention

A key pathogenic mechanism of Salmonella is the organism's ability to invade the cells of the intestinal epithelium. Electron microscopic studies of Salmonella-infected laboratory animals (Takeuchi, A., Am J Pathol (1967) 50:109-136) and cultured cells (Finlay & Falkow, Mol Microbiol (1989) 3:1833-1841; Kohbata, S. et al., Microbiol Immunol (1986) 30:1225-1237) have shown that these organisms enter epithelial cells after transient disruption of the surface microvilli. Bacteria are later seen within endocytic vacuoles, in which, in some instances, they undergo replication. It is currently believed that Salmonella strains, unlike other invasive bacteria, such as Shigella spp. (Bernardini, J.L., et al., Proc Natl Acad Sci USA (1989) 86:3867-3871; Makino, S., et al., Cell (1986) 46:551-555; Sansonetti, P., et al., Infect Immun (1986) 51:461-469) or Listeria spp. (Mounier, J., et al., Infect Immun (1990) 58:1048-1058; Tilney & Portnoy, J Cell Biol (1989) 109:1597-1608) do not leave the endocytic vesicle to gain access to the cytosol. Instead, it appears that Salmonella strains

translocate through the epithelial cell in membrane-bound vesicles to later exit at the basolateral surface of the epithelium (Finlay & Falkow, Mol Microbiol (1989) 3:1833-1841; Kohbata, S. et al. Microbiol Immunol (1986) 30:1225-1237; Takeuchi, A., Am J Pathol (1967) 50:109-136).

There are three primary species of Salmonella (S. typhi, S. choleraesuis and S. enteritidis) and hundreds of serovars that infect a variety of different hosts (Hook, E.W., Principles and Practice of Infectious Diseases, 2nd ed., John Wiley and Sons, New York (1985) (G.L. Mandell, R.G. Douglas, Jr. and Benner, J.E., eds.) Some species and serovars are host adapted (e.g., S. typhi and S. gallinarum), while others can infect a variety of hosts (e.g., S. typhimurium and S. enteritidis). Although invasion of epithelial cells appears to be a common essential virulence factor of all Salmonella strains, it is not known whether all species and serovars interact with eukaryotic cells in a similar fashion. In fact, there is some evidence to suggest that differences may exist. Rough strains of S. choleraesuis and S. typhi are deficient in their ability to enter cultured mammalian cells (Finlay, B.B., et al., Mol Microbiol (1988) 2:757-766; Mroczenski-Wildey, M.J., et al., Microb Pathog (1989) 6:143-152), while S. typhimurium rough strains are not (Kihlstrom & Edebo, Infect Immun (1976) 14:851-857; Kihlstrom & Nilsson, Acta Pathol Microbiol Scand (1977) 85:322-328). In addition, Elsinghorst, E.A., et al., Proc Natl Acad Sci USA (1989) 86:5173-5177, cloned a chromosomal region of S. typhi that conferred upon Escherichia coli HB101 the ability to enter Henle-407 cells. The same chromosomal region from S. typhimurium did not confer invasive properties upon E. coli, suggesting that the S. typhi-homologous genes are

either defective or nonfunctional in S. typhimurium or are not expressed in E. coli.

Recently a group of genes was cloned (invA, B, C, and D) that allow S. typhimurium to enter cultured epithelial cells (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). The invA, B, and C genes are arranged in the same transcriptional unit, while the invD gene is located downstream in a different transcriptional unit. Virulent strains of S. typhimurium carrying defined mutations in invA (and therefore unable to express invA, B, and C) had higher 50% lethal doses than their parent strains when administered orally to mice and were deficient in their ability to colonize Peyer's patches and the small intestinal wall. In contrast, invA mutants were fully virulent when administered intraperitoneally, suggesting that the inv genes are only needed for the display of virulence when S. typhimurium is administered by the natural route of entry (id.). In addition, studies conducted with transcriptional and translational fusions of reporter genes to invA have established that the expression of the inv genes is regulated by changes in DNA supercoiling as a consequence of a variety of environmental signals, such as osmolarity, temperature, and oxygen tension (Galan & Curtiss, Infect Immun (1990) 58:1879-1885). Conditions found in the gut are optimal for the expression of these genes.

Disclosure of the Invention

It has now been found that polynucleotide sequences present in the Salmonella typhimurium inv genes can be used as universal probes or primers for the detection of almost any Salmonella species, strain or serotype. These sequences are specific for Salmonella and do not react with other closely related enteric

bacteria. As such, these sequences can serve as important clinical diagnostic agents.

Accordingly, in one embodiment, the subject invention is directed to an oligomer capable of hybridizing to a Salmonella polynucleotide sequence in an analyte strand. The oligomer comprises at least 8 contiguous nucleotides derived from a Salmonella typhimurium inv gene.

In another embodiment, the present invention is directed to a method for detecting the presence or absence of a Salmonella polynucleotide sequence in an analyte strand. The method comprises:

(a) providing at least one oligomer capable of hybridizing to a Salmonella target sequence in a target region of an analyte strand, the at least one oligomer comprising a Salmonella targeting sequence complementary to at least 8 contiguous nucleotides derived from a Salmonella typhimurium inv gene; and

(b) incubating the analyte strand with the at least one oligomer under conditions which allow specific hybrid duplexes to form between the Salmonella target sequence and the Salmonella targeting sequence, thereby detecting the presence or absence of the Salmonella polynucleotide sequence.

In a preferred embodiment, the method uses a set of oligomers which are primers for the polymerase chain reaction method and which flank the target region. The target region is amplified via the polymerase chain reaction.

In yet another embodiment, the subject invention is directed to a kit for detecting the presence or absence of a Salmonella polynucleotide sequence in an analyte strand. The kit comprises an oligomer as described above, packaged in a suitable container.

In particularly preferred embodiments, the oligomer is derived from the invA, B, C, and/or D genes, or the invABC operon.

These and other embodiments of the present invention will readily occur to those of ordinary skill in the art in view of the disclosure herein.

Brief Description of the Figures

Figure 1 depicts the nucleotide sequence of the invA gene of S. typhimurium.

Figure 2 shows the restriction maps for the recombinant plasmids from which DNA probes were derived. Figure 2A shows the restriction map for plasmid pYA2220. Figure 2B shows the restriction map for plasmid pYA2219. The positions of the invABC genes and the direction of transcription are indicated by horizontal arrows. The position of the invD gene is indicated by the heavy line underneath pYA2219 and is contained within the 2.4-kb EcoRI fragment of pYA2219. The region shown by the heavy line was determined by TnphoA mutagenesis (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). B, BamHI; Bg, BglII; C, ClaI; E, EcoRI; H, HindIII; Hn, HincII; P, PstI; Pv, PvuII; S, SalI.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell culture, molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual (Second edition, 1989); DNA Cloning (1985) Vols. I and II, D.N. Glover (ed.); Nucleic Acid Hybridization (1984), B.D. Hames, et al. (eds.); Perbal, B., A Practical Guide to

Molecular Cloning (1984); Methods in Enzymology (the series), Academic Press, Inc.; Vectors: A Survey of Molecular Cloning Vectors and Their Uses (1987), R.L. Rodriguez, et al., (eds.), Butterworths; and Miller, J.H., et al., Experiments in Molecular Genetics (1972) Cold Spring Harbor Laboratory.

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference.

A. Definitions

By "Salmonella" is meant any bacterium either currently classified or later identified in the genus Salmonella. The salmonellae are motile rods that characteristically ferment glucose and mannose without producing gas but do not ferment lactose or sucrose. The group includes three primary species, S. typhi, S. choleraesuis and S. enteritidis, and hundreds of serovars that infect a variety of different hosts. Some serotypes are primarily infective for humans, however the vast majority of salmonellae are chiefly pathogenic in animals that can serve as a source for human infection i.e. poultry, pigs, rodents, cattle, pets and several others. For a review of the salmonellae, see Hook, E.W., Principles and Practice of Infectious Diseases, 2nd ed., John Wiley and Sons, New York (1985) (G.L. Mandell, R.G. Douglas, Jr. and Benner, J.E., eds.).

By a "Salmonella typhimurium inv gene" is meant any of the group of genes found in S. typhimurium which is responsible for the ability of S. typhimurium to enter cultured epithelial cells as determined by conventional assays, including the tissue culture cell assay described in the examples. At least four genes have been found to be involved in the invasive phenotype -- invA, invB, invC and invD. In some instances, as depicted in Figure 2,

the invA, B and C genes are arranged in the same transcriptional unit (called the "invABC operon" herein) and invD is located downstream of this cluster in an independent transcriptional unit.

As used herein, a nucleotide seqence "derived from" a designated sequence refers to a nucleotide sequence capable of specifically hybridizing to a Salmonella sequence and which is comprised of a sequence of approximately at least about 8 nucleotides, preferably at least about 10-12 nucleotides, more preferably at least about 15-20 nucleotides corresponding to a region of the designated nucleotide sequence. "Corresponding" means homologous to or complementary to the designated sequence. Hybridization techniques for determining the complementarity of nucleic acid sequences are known in the art, and are discussed infra. In addition, mismatches of duplex polynucleotides formed by hybridization can be determined by known techniques, including for example, digestion with a nuclease such as S1 that specifically digests single-stranded areas in duplex polynucleotides. Regions from which typical DNA sequences of the subject invention may be "derived" include any of the S. typhimurium inv genes, including but not limited to the invA, B, or C genes, either individually or as a transcriptional unit, and/or the invD gene.

The derived nucleotide sequence is not necessarily physically derived from the nucleotide sequence shown, but may be generated in any manner, including for example, chemical synthesis or DNA replication or reverse transcription or transcription. In addition, combinations of regions corresponding to that of the designated sequence may be modified in ways known in the art to be consistent with an intended use.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, (3) does not occur in nature, or (4) is not in the form of a library.

The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), those with intercalators (e.g., acridine, psoralen, etc.), those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified linkages (e.g., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

As used herein, the "sense strand" of a nucleic acid contains the sequence that has sequence homology to that of mRNA. The "anti-sense strand" contains a

sequence which is complementary to that of the "sense strand".

The term "primer" as used herein refers to an oligomer which is capable of acting as a point of initiation of synthesis of a polynucleotide strand when placed under appropriate conditions. The primer will be completely or substantially complementary to a region of the polynucleotide strand to be copied. Thus, under conditions conducive to hybridization, the primer will anneal to the complementary region of the analyte strand. Upon addition of suitable reactants, (e.g., a polymerase, nucleotide triphosphates, and the like), the primer is extended by the polymerizing agent to form a copy of the analyte strand. The primer may be single-stranded, or alternatively may be partially or fully double-stranded.

The terms "analyte polynucleotide" and "analyte strand" refer to a single- or double-stranded nucleic acid molecule which is suspected of containing a target sequence, and which may be present in a biological sample.

As used herein, the term "oligomer" refers to primers and to probes. The term oligomer does not connote the size of the molecule. An oligomer may comprise an entire transcript. Alternatively, an oligomer may comprise only part of a gene. If so, the oligomer will generally be no greater than 1000 nucleotides, more typically no greater than 500 nucleotides, even more typically no greater than 250 nucleotides; it may be no greater than 100 nucleotides, and may be no greater than 75 nucleotides, and also may be no greater than 50 nucleotides in length.

As used herein, the term "probe" refers to a structure comprised of a polynucleotide which forms a hybrid structure with a target sequence, due to complementarity of at least one sequence in the probe

with a sequence in the target region. The polynucleotide regions of probes may be composed of DNA, and/or RNA, and/or synthetic nucleotide analogs. Preferably the probe does not contain a sequence complementary to sequence(s) used to prime the polymerase chain reaction (PCR).

As used herein, the term "target region" refers to a region of the nucleic acid which is to be amplified and/or detected. The term "target sequence" refers to a sequence with which a probe or primer will form a stable hybrid under desired conditions.

The term "targeting polynucleotide sequence" as used herein, refers to a polynucleotide sequence which is comprised of nucleotides which are complementary to a target nucleotide sequence; the sequence is of sufficient length and complementarity with the target sequence to form a duplex which has sufficient stability for the purpose intended.

The term "coupled" as used herein refers to attachment by covalent bonds or by strong non-covalent interactions (e.g., hydrophobic interactions, hydrogen bonds, etc.). Covalent bonds may be, for example, ester, ether, phosphoester, amide, peptide, imide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like.

The term "support" refers to any solid or semi-solid surface to which a desired binding partner may be anchored. Suitable supports include glass, plastic, metal, polymer gels, and the like, and may take the form of beads, wells, dipstics, membranes, and the like.

The term "label" as used herein refers to any atom or moiety which can be used to provide a detectable (preferably quantifiable) signal, and which can be attached to a polynucleotide or polypeptide.

As used herein, the term "label probe" refers to an oligomer which is comprised of a targeting

polynucleotide sequence, which is complementary to a target sequence to be detected in the analyte polynucleotide. This complementary region is of sufficient length and complementarity to the target sequence to afford a duplex comprised of the "label probe" and the "target sequence" to be detected by the label. The oligomer is coupled to a label either directly, or indirectly via a set of ligand molecules with high specificity for each other. Sets of ligand molecules with high specificity are known in the art and include for example biotin and avidin or streptavidin, IgG and protein A, other numerous known receptor-ligand couples, and complementary polynucleotide strands.

As used herein, a "biological sample" refers to a sample of tissue or fluid isolated from a vertebrate subject, including but not limited to, for example, blood, plasma, serum, stool, urine, bone marrow, bile, spinal fluid, lymph fluid, the external sections of the skin, respiratory, intestinal, and genitourinary tracts, tears, saliva, milk, blood cells, organs, and also samples of *in vitro* cell culture constituents (including but not limited to conditioned medium resulting from the growth of cells in cell culture medium, putatively *Salmonella* infected cells, recombinant cells, and cell components).

B. General Methods

The present invention is based on the discovery that the *S. typhimurium inv* genes can be used as universal probes and primers for the general detection of *Salmonella*. Using the techniques described herein, four *inv* genes, *invA*, *invB*, *invC*, and *invD*, have been isolated and the *invA* gene sequenced (Figure 1). The distribution of these genes among different *Salmonella* species and serovars, as well as other enteric organisms, has been

investigated using Southern and colony blot hybridization analyses as well as PCR amplification assays. As explained above, the invA, B, and C genes occur as a single transcriptional unit with the invD gene found downstream of this operon. The genes are unique to the salmonellae, and as such, provide an eloquent means for detecting the presence of any Salmonella organism in a biological sample.

Utilizing the above-described inv genes, oligomers can be constructed which are useful as reagents for detecting Salmonella polynucleotides in biological samples. For example, DNA oligomers of about 8-10 nucleotides or larger, can be synthesized using standard techniques. These oligomers, in turn, can be used as hybridization probes and amplification primers to detect the presence or absence of Salmonella DNA in, for example, blood, blood fractions, sera, bone marrow, bile, urine, stool specimens, saliva or other biological samples (as defined above) from vertebrate subjects suspected of harboring Salmonella. In addition, cultures suspected of containing Salmonella can also be tested for the presence or absence of the organism therein.

The novel oligomers described herein also enable further characterization of the Salmonella genome as well as the elucidation of the mode of invasion of these organisms. The oligomers can also be used for identifying new Salmonella strains and serotypes. Since the described probes and primers are specific for Salmonella, organisms suspected of belonging to the Salmonella genus can be tested for their ability to hybridize with these oligomers. If hybridization occurs, these bacteria can be further characterized.

Oligomer Probes and Primers

As explained above, oligomers of approximately 8 nucleotides or more can be prepared which specifically hybridize with Salmonella target sequences. These oligomers can serve as probes for the detection (including isolation and/or labeling) of polynucleotides which contain Salmonella nucleotide sequences, and/or as primers for the transcription and/or replication of targeted Salmonella sequences. The oligomers contain a targeting polynucleotide sequence (as defined above), which is comprised of nucleotides which are complementary to a target Salmonella nucleotide sequence. The sequence is of sufficient length and complementarity with the Salmonella sequence to form a duplex with sufficient stability for the purpose intended. For example, if the purpose is the isolation, via immobilization, of an analyte containing a target Salmonella sequence, the oligomers should include a polynucleotide region of adequate length and complementarity such that the analyte can be immobilized on a solid surface under the isolation conditions.

If the oligomers are to serve as primers for the transcription and/or replication of target Salmonella sequences in an analyte polynucleotide, they should contain a polynucleotide region of sufficient length and complementarity to the targeted Salmonella sequence to allow the polymerizing agent to continue replication from the primers which are in stable duplex form with the target sequence, under the polymerizing conditions.

The oligomers may contain a minimum of about 4-6 contiguous nucleotides which are complementary to the targeted Salmonella sequence, usually a minimum of about 8 continguous nucleotides, and preferably a minimum of about 14 contiguous nucleotides which are complementary

to the targeted Salmonella sequence. However, a minimum of about 20 nucleotides or more appears optimal.

Suitable Salmonella nucleotide targeting sequences may be comprised of contiguous sequences of nucleotides from any of the inv genes including invA, invB, invC, and invD. As explained above, the sequences used need not represent the complete gene, so long as they are of sufficient length to hybridize to a Salmonella target sequence. Particularly useful are probes derived from the invA gene sequence depicted in Figure 1. It has been found, using polymerase chain reaction (PCR) technology, that primers from this sequence are able to specifically amplify Salmonella gene sequences from hundreds of strains of the organism while failing to react with other closely related enteric bacteria. Other particularly suitable sequences include those derived from the invABC operon. Such sequences can include all of the operon, such as probe 2 in Figure 2A, or truncated forms of the same, such as probe 1 in Figure 2A. Again, such probes have been found to be highly specific for the salmonellae. Also of use are probes derived from the invD gene, such as probe 3 of Figure 2B herein. One of skill in the art can readily devise other useful probes based on the inv genes with reference to the disclosure herein.

The oligomer may contain, in addition to the target sequences, nucleotide sequences or other moieties. For example, if the oligomers are used as primers for the amplification of Salmonella sequences via PCR, they may include sequences which, when in duplex, form restriction enzyme sites which facilitate the cloning of the amplified sequences.

The preparation of the oligomers is by routine methods, including, for example, excision, transcription, or chemical synthesis.

Hybridization Assays

To detect the presence of Salmonella polynucleotides in a biological sample, the speciman to be analyzed may be treated, if desired, to extract the nucleic acids contained therein. The resulting nucleic acid from the sample is subjected to gel electrophoresis or other size separation techniques. Alternatively, the nucleic acid sample may be dot blotted without size separation.

In order to form hybrid duplexes with the targeting sequence of the probe, the targeted region of the analyte nucleic acid must be in single stranded form. Denaturation can be carried out by various techniques known in the art. Subsequent to denaturation, the analyte nucleic acid and probe are incubated under conditions which promote stable hybrid formation of the target sequence in the probe with the putative targeted sequence in the analyte, and the resulting duplexes containing the probe(s) are detected.

Detection of the resulting duplex, if any, is usually accomplished by the use of labeled probes. Alternatively, the probe may be unlabeled, but may be detectable by specific binding with a ligand which is labeled, either directly or indirectly. Suitable labels, and methods for labeling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies, and the like.

The region of the probes which are used to bind to the analyte can be made completely complementary to an inv gene. Therefore, high stringency conditions can be used in order to prevent false positives. The stringency of hybridization is determined by a number of factors

during hybridization and during the washing procedure, including temperature, ionic strength, length of time, and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (1989).

Variations of this basic scheme which are known in the art, including those which facilitate separation of the duplexes to be detected from extraneous materials and/or which amplify the signal from the labeled moiety, may also be used. A number of these variations are reviewed in, for example: Matthews and Kricka (1988), Analytical Biochemistry 169:1; Landegren et al. (1988), Science 242:229; and Mittlin (1989), Clinical chem. 35:1819. These and the following publications describing assay formats are hereby incorporated by reference in their entirety. Probes suitable for detecting Salmonella in these assays are comprised of sequences which hybridize with target Salmonella polynucleotide sequences to form duplexes with the analyte strand, wherein the duplexes are of sufficient stability for detection in the specified assay system.

A suitable variation is, for example, one which is described in U.S. Patent No. 4,868,105, issued Sept. 9, 1989, and in EPO Publication No. 225,807 (published June 16, 1987). These publications describe a solution phase nucleic acid hybridization assay in which the analyte nucleic acid is hybridized to a labeling probe set and to a capturing probe set. The probe-analyte complex is coupled by hybridization with a solid-supported capture probe that is complementary to the capture probe set. This permits the analyte nucleic acid to be removed from solution as a solid phase complex. Having the analyte in the form of a solid phase complex facilitates subsequent separation steps in the assay. The labeling probe set is complementary to a

labeled probe that is bound through hybridization to the solid phase/analyte complex.

It is possible that the Salmonella inv sequences will be present in serum of infected subjects at relatively low levels. Accordingly, amplification techniques may be used in the hybridization assays in order to increase the sensitivity thereof. Such techniques are known in the art. For example, the Enzo Biochemical Corporation "Bio-Bridge" system uses terminal deoxynucleotide transferase to add unmodified 3'-poly-dT-tails to a DNA probe. The poly dT-tailed probe is hybridized to the target nucleotide sequence, and then to a biotin-modified poly-A. PCT Publication 84/03520 and EP Publication No. 124221 describe a DNA hybridization assay in which: (1) analyte is annealed to a single-stranded DNA probe that is complementary to an enzyme-labeled oligonucleotide; and (2) the resulting tailed duplex is hybridized to an enzyme-labeled oligonucleotide. EPA 204510 describes a DNA hybridization assay in which analyte DNA is contacted with a probe that has a tail, such as a poly-dT tail, an amplifier strand that has a sequence that hybridizes to the tail of the probe, such as a poly-A sequence, and which is capable of binding a plurality of labeled strands. A type of hybridization assay which is described in EPO Publication No. 317,077 (published May 24, 1989), which should detect sequences at the level of approximately 10^6/ml, utilizes nucleic acid multimers which bind to single-stranded analyte nucleic acid, and which also bind to a multiplicity of single-stranded labeled oligonucleotides.

The Polymerase Chain Reaction

A particularly desirable technique for the detection of Salmonella using polynucleotides derived

from the inv genes involves amplification of the target Salmonella sequences using the polymerase chain reaction (PCR) technique described by Saiki et al. (1986), by Mullis, U.S. Patent No. 4,683,195, and by Mullis et al. U.S. Patent No. 4,683,202, the disclosures of which are incorporated herein by reference in their entirety. Amplification may be prior to, or preferably subsequent to, purification of the Salmonella target sequence. Amplification may be utilized in conjunction with the assay methods described above. The PCR method uses primers and probes derived from the information provided herein concerning the Salmonella inv genes.

Generally, in the PCR technique, short oligonucleotide primers are prepared which match opposite ends of a desired sequence. The sequence between the primers need not be known. A sample of polynucleotide is extracted and denatured. Strand separation may be accomplished by any suitable denaturing method, including physical, chemical, or enzymatic means, which are known to those of skill in the art. A commonly used method, which is physical, involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80°C to about 105°C, for times ranging from about 1 to 10 minutes.

Following denaturation, the analyte strand is hybridized with oligonucleotide primers which are present in molar excess. Polymerization is catalyzed by a template- and primer-dependent polymerase in the presence of deoxynucleotide triphosphates or nucleotide analogs (dNTPs). This results in two "long products" which contain the respective primers at their 5'-termini, covalently linked to the newly synthesized complements of the original strands. The replicated DNA is again denatured, hybridized with oligonucleotide primers,

returned to polymerizing conditions, and a second cycle of replication is initiated. The second cycle provides the two original strands, the two long products from cycle 1, and two "short products" replicated from the long products. The short products contain sequences (sense or antisense) derived from the target sequence, flanked at the 5'- and 3'-termini with primer sequences. On each additional cycle, the number of short products is replicated exponentially. Thus, this process causes the amplification of a specific target sequence.

The primers are selected so that their relative positions along a duplex sequence are such that an extension product synthesized from one primer, when it is separated from its template (complement), serves as a template for the extension of the other primer to yield a replicate chain of defined length.

The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and source of the primer and use of the method. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains about 15-45 nucleotides, although it may contain more or fewer nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template.

The primers used herein are selected to be "substantially" complementary to the different strands of

each specific sequence to be amplified. Therefore, the primers need not reflect the exact sequence of the template, but must be sufficiently complementary to selectively hybridize with their respective strands. For example, a non-complementary nucleotide fragment may be attached to the 5'-end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer has sufficient complementarity with the sequence of one of the strands to be amplified to hybridize therewith, and to thereby form a duplex structure which can be extended by the polymerizing means. The non-complementary nucleotide sequences of the primers may include restriction enzyme sites. Appending a restriction enzyme site to the end(s) of the target sequence would be particularly helpful for cloning of the target sequence.

It will be understood that "primer", as used herein, may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding the terminal sequence(s) of the target region to be amplified. Hence, a "primer" includes a collection of primer oligonucleotides containing sequences representing the possible variations in the sequence or includes nucleotides which allow a typical basepairing. One of the primer oligonucleotides in this collection will be homologous with the end of the target sequence.

Particularly useful primers for the amplification of Salmonella inv sequences are those derived from the invA gene depicted in Figure 1. A set of primers based on the invA gene sequence and used in the PCR technique is described in the examples herein. These primers specifically amplified 99.4% of the 636

Salmonella strains tested and failed to react with other non-Salmonella strains. One skilled in the art can easily devise other primers for use in the PCR method by reference to the instant disclosure.

The oligonucleotide primers may be prepared by any suitable method. Methods for preparing oligonucleotides of a specific sequence are known in the art, and include, for example, cloning and restriction of appropriate sequences, and direct chemical synthesis. Chemical synthesis methods may include, for example, the phosphotriester method described by Narang et al. (1979), the phosphodiester method disclosed by Brown et al. (1979), the diethylphosphoramidate method disclosed in Beaucage et al. (1981), and the solid support method in U.S. Patent No. 4,458,066.

The primers may be labeled, if desired, by incorporating means detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means.

Template-dependent extension of the oligonucleotide primer(s) is catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleotide triphosphates (dATP, dGTP, dCTP and dTTP) or analogs, in a reaction medium which is comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. Known DNA polymerases include, for example, E. coli DNA polymerase I or its Klenow fragment, T_4 DNA polymerase, and Taq DNA polymerase. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are known in the art.

The products of the synthesis are duplex molecules consisting of the template strands and the primer extension strands, which include the target sequence. These products, in turn, serve as template for

another round of replication. In the second round of replication, the primer extension strand of the first cycle is annealed with its complementary primer. Synthesis yields a "short" product which is bounded on both the 5'- and the 3'-ends by primer sequences or their complements. Repeated cycles of denaturation, primer annealing, and extension result in the exponential accumulation of the target region defined by the primers. Sufficient cycles are run to achieve the desired amount of polynucleotide containing the target region of nucleic acid. The desired amount may vary, and is determined by the function which the product polynucleotide is to serve.

The PCR method can be performed in a number of temporal sequences. For example, it can be performed step-wise, where after each step new reagents are added, or in a fashion where all of the reagents are added simultaneously, or in a partial step-wise fashion, where fresh reagents are added after a given number of steps.

In a preferred method, the PCR reaction is carried out as an automated process which utilizes a thermostable enzyme. In this process the reaction mixture is cycled through a denaturing region, a primer annealing region, and a reaction region. A machine may be employed which is specifically adapted for use with a thermostable enzyme, which utilizes temperature cycling without a liquid handling system, since the enzyme need not be added at every cycle.

After amplification by PCR, the target polynucleotides are detected by hybridization with a probe polynucleotide which forms a stable hybrid with that of the target sequence under stringent to moderately stringent hybridization and wash conditions. If it is expected that the probes will be completely complementary (i.e., about 99% or greater) to the target sequence,

stringent conditions can be used. If some mismatching is expected, the stringency of hybridization may be lessened. However, conditions are chosen which rule out nonspecific/adventitious binding. Conditions which affect hybridization, and which select against nonspecific binding are known in the art, and are described in, for example, Sambrook et al. (1989). Generally, lower salt concentration and higher temperature increase the stringency of binding. For example, it is usually considered that stringent conditions include incubation in solutions which contain approximately 0.1 X SSC, 0.1% SDS, at about 65°C incubation/wash temperature, and moderately stringent conditions are those where incubation occurs in solutions which contain approximately 1-2 X SSC, 0.1% SDS and about 50°-65°C incubation/wash temperature. Low stringency conditions are 2 X SSC and about 30°-50°C.

Probes for use in the hybridization reaction may be derived from the Salmonella inv genes as described above. The probes may be of any suitable length which span the target region, but which exclude the primers, and which allow specific hybridization to the target region. If there is to be complete complementarity, i.e., if the strain contains a sequence identical to that of the probe, since the duplex will be relatively stable even under stringent conditions, the probes may be short, i.e., in the range of about 10-30 base pairs. If some degree of mismatch is expected with the probe, the probe may be of greater length, since length seems to counterbalance some of the effect of the mismatch(es).

The probe nucleic acid having a sequence complementary to the target sequence may be synthesized using similar techniques described above for the synthesis of primer sequences. If desired, the probe may be labeled. Appropriate labels are also described above.

The presence of the target sequence in a biological sample is detected by determining whether a hybrid has been formed between the Salmonella polynucleotide probe and the nucleic acid subjected to the PCR amplification technique. Methods to detect hybrids formed between a probe and a nucleic acid sequence are known in the art. For example, for convenience, an unlabeled sample may be transferred to a solid matrix to which it binds, and the bound sample subjected to conditions which allow specific hybridization with a labeled probe. The solid matrix is than examined for the presence of the labeled probe. Alternatively, if the sample is labeled, the unlabeled probe is bound to the matrix, and after the exposure to the appropriate hybridization conditions, the matrix is examined for the presence of label. Other suitable hybridization assays are described supra.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples, which are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

Deposits of Strains Useful in Practicing the Invention

A deposit of biologically pure cultures of the following strains were made with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland. The accession number indicated was assigned after successful viability testing, and the requisite fees were paid. Access to said cultures will be available during pendency of the patent application to one determined by the Commissioner to be entitled thereto under 37 CFR 1.14 and 35 USC 122. All restriction on availability of said cultures to the public will be ir-

revocably removed upon the granting of a patent based upon the application. Moreover, the designated deposits will be maintained for a period of thirty (30) years from the date of deposit, or for five (5) years after the last request for the deposit; or for the enforceable life of the U.S. patent, whichever is longer. Should a culture become nonviable or be inadvertently destroyed, or, in the case of plasmid-containing strains, loose its plasmid, it will be replaced with a viable culture(s) of the same taxonomic description.

Strain	Deposit Date	ATCC No.
pYA2220 in E. coli	August 22, 1991	

C. Experimental

Materials and Methods

Bacterial strains, tissue culture cells, and growth conditions. The strains used in the experimental section are listed in Tables 1 and 2. The genotypes and sources of S. typhimurium strains LT2-Z, SR-11, SL1344, and χ3456 have been described (Gulig & Curtiss, Infect Immun (1987) 55:2891-2901). S. typhimurium strain DB4673 is an isolate of TS736 (Palva & Liljeström, Mol Gen Genet (1981) 181:153-157) and was obtained from D. Botstein (Massachusetts Institute of Technology). E. coli strains CC118 (Manoil & Beckwith, Proc Natl Acad Sci USA (1985) 82:8129-8133), JC7623 (Winans, S.C., et al., J Bacteriol (1985) 161:1219-1221), and χ2819 (Jacobs, W.R., et al., Infect Immun (1986) 52:101-109) have also been described. Bacteria were grown in L broth or on L agar plates (Lennox, E.S., Virology (1955) 1:190-206). When appropriate, 30 μg of kanamycin per ml was added to the growth media. Human intestinal Henle-407 cells were grown as described elsewhere (Galan and Curtiss, 1990).

Bacteriophage transductions. Bacteriophage P22HTint-mediated transductions were performed as indicated previously (Schmeiger, H., Mol Gen Genet (1972) 119:74-88).

DNA isolation and probe preparation. Plasmid DNA was isolated by the method of Birnboim, H.C., and Doly, J., Nucleic Acids Res (1979) 7:1513-1523. Total cell DNA was isolated as follows. Bacterial strains were grown overnight in L broth at 37°C in a rotating wheel. Cultures (5 ml) were washed twice in buffered saline containing 0.1% (wt/vol) gelatin and resuspended in 1 ml of cold lysing buffer (50 mM glucose-10 mM EDTA-25 mM Tris-HCl (pH 8.0)-1 mg of lysozyme (Sigma, St. Louis, MO) per ml. The suspensions were incubated for 10 min on ice, and EDTA (0.250 ml of a 0.5 M solution) and lauryl sarcosine (0.125 ml of a 10% (wt/vol) solution) were added. Samples were incubated for 20 min in a 55°C water bath. Cell lysates were extracted with phenol once, phenol-chloroform (1:1) twice, chloroform once, and ether twice. DNA samples were ethanol precipitated, resuspended in 0.5 ml of 10 mM Tris (pH 8.0)-1 mM EDTA, digested with RNase A (50 μg/ml) (Sigma) for 15 min at room temperature, and stored at -20°C until further use. Restriction endonuclease enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and International Biotechnologies, Inc. (New Haven, Conn.) and used in accordance with manufacturer instructions. DNA probes were prepared as follows. Plasmid DNA was digested with the appropriate enzymes, and DNA fragments were separated by electrophoresis on a 0.7% agarose gel. The DNA fragments of interest were isolated with Geneclean (Bio 101, La Jolla, Calif.), denatured by being heated at 90°C for 5 min, and labeled with [α-^{32}P]ATP (Amersham Corp., Arlington Heights, Ill.) by use of a

random primer DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.)

Southern and colony blot hybridization analyses. Total-cell DNA samples were digested with EcoRI and PvuII. DNA fragments were separated on a 0.7% agarose gel and transferred to nylon membranes (GeneScreen Plus; Dupont, Wilmington, Del.) by the method of Southern (Southern, E.M., J Mol Biol (1975) 98:503-517). In high-stringency hybridization experiments, membranes were prehybridized for 4 h at 37°C in 50% formamide-1% sodium dodecyl sulfate (SDS)-1 M sodium chloride-10% dextran sulfate. Hybridization was carried out at 37°C in the same solution containing 250 μg of denatured salmon sperm DNA per ml and boiled (10 min at 100°C) probe for 15 h. Membranes were washed two times for 5 min each time in 2x SSC (1x SSC is 150 mM sodium chloride-15 mM sodium citrate) at room temperature, two times for 30 min each time in 2x SSC-1% SDS at 65°C, and two times for 30 min each time in 0.1x SSC at room temperature. In low-stringency experiments, prehybridization and hybridization were carried out under similar conditions, except that 20% formamide was used and washes were performed at 55°C in buffer containing 0.1% SDS. Membranes were air dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). Membranes were reused after being washed with 0.4 N sodium hydroxide at 42°C for 2 to 5 h and with 0.1x SSC-0.1% SDS-0.2 M Tris-HCl (pH 7.5) at 42°C for 2 h. Washed blots were exposed to X-Omat AR film to verify successful washing. Colony blots were prepared as described elsewhere (Maniatis, T., et al., Molecular Cloning: A Laboratory Manual (1982), Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) and hybridized as described above. These blots were also reused after being washed as described above. Stringency of hybridization was calculated with the assumption that

there is a drop of 1°C in melting temperature (T_m) for every 1% base-pair mismatch (Bonner, T.I., et al., J Mol Biol (1973) 81:123-135) by use of the formula T_m = 81.5 + 16.6 log M + 041 (percent G + C content) - (500/n), where n is the length of the probe in base pairs and M is the molarity. On the basis of sequencing data, the probe is 48% G + C; therefore high- and low-stringency experiments would allow 22 and 32% base-pair mismatches, respectively.

TABLE 1. Salmonella strains used in genetic manipulations

Strain	Species or Serovar	Relevant Genotype	Description, reference, or source
SL1344	S. typhimurium	Wild type	21
SB103	S. typhimurium	invA::kan	invA deriv. SL1344 (Ginocchio & Galan)
Ty2	S. typhi	Wild type	Obt'd from D. Hone (Univ. of Md.)
SB129	S. typhi	invA::kan	P22HTint (SB103) ⇒ Ty2
ISP2825	S. typhi	Wild type	Obt'd from D. Hone (Univ. of Md.)
SB130	S. typhi	invA::kan	P22HTint (SB103) ⇒ ISP2825
7193	S. enteritidis	Wild type	Obt'd from D. Hone (Univ. of Md.)
SB131	S. enteritidis	invA::kan	P22HTint (SB103) ⇒ 7193
Stock	S. gallinarum	Wild type	Obt'd from An. Disease Ctr.(Ames,Iowa)
SB132	S. gallinarum	invA::kan	P22HTint (SB103) ⇒ Stock
Lane	S. dublin	Wild type	8
SB133	S. dublin	invA::kan	P22HTint (SB103) ⇒ Lane

TABLE 2. Bacterial strains tested for hybridization to the invABC and invD probes

	O group or serotype	No. of isolates tested	Origin, description, or reference
Salmonella species or serovars			
S. typhimurium	B	20	Human, bovine, equine Porcine, avian
S. enteritidis	D1	15	Human, avian
S. dublin	D	4	Human, bovine
S. typhi	D1	3	Human
S. choleraesuis	C1	3	Porcine
S. gallinarum	D	3	Avian
S. pullorum	D1	2	Avian
S. arizonae	61	2	Avian
S. anatum	E1	3	Human
S. infantis	C1	3	Human
S. hadar	C2	2	Human
S. heidelberg	B	2	Human
S. london	E1	2	Human
S. panama	D1	2	Human
S. bovismorbificans	C2	2	Human
S. manhattan	C2	2	Human

Table 2 (continued)

S. agona	B	1	Human	
S. albany	C3	1	Human	
S. branderup	C1	1	Human	
S. brandenburg	C1	1	Human	
S. bredeney	B	1	Human	
S. cerro sieburg	K	1	Human	
S. derby	B	1	Human	
S. glostrup	C2	1	Human	
S. give	E1	1	Human	
S. montevideo	C1	1	Human	
S. nienstedten	C4	1	Human	
S. othmarschen	C1	1	Human	
S. schwarzengrund	B	1	Human	
S. thompson	C1	1	Human	
S. veile	E1	1	Human	
S. virchow	C1	1	Human	
S. java	B	1	Human	
S. duisburg	E1	1	Human	
S. tennessee	C1	1	Human	
S. ohio	C1	1	Human	
S. newport	C2	1	Human	

E. coli areina[a]		
χ289		Wild-type K-12
EIEC1	O28ac:H-	Enteroinvasive
EIEC5	O28ac:H-	Enteroinvasive
EIEC10	O29:H-	Enteroinvasive
EIEC15	O29:-	Enteroinvasive
EIEC19	O112ac:H-	Enteroinvasive
EIEC22	O124:H-	Enteroinvasive
EIEC32	O136:H-	Enteroinvasive
EIEC36	O136:H-	Enteroinvasive
EIEC42	O143:H-	Enteroinvasive
EIEC50	O144:H-	Enteroinvasive
EIEC55	O152:H-	Enteroinvasive
EIEC65	O164:H-	Enteroinvasive
EIEC74	O167:H-	Enteroinvasive
E2348/69	O127:K63:H6	Enteropathogenic
E851/71	O142	Enteropathogenic
E2831/70	O142	Enteropathogenic
Other bacterial strains		
Shigella flexneri 2a BS185		34
Yersinia pseudotuberculosis YPIII		6
Yersinia enterocolitica 8081		43

[a] All but χ289 were obtained from J. Kaper (Center for Vaccine Development, University of Maryland).

Example 1

Tissue Culture Cell Invasion by Salmonella Strains

A total of 91 Salmonella strains (Table 2) were tested for their ability to invade cultured Henle-407 cells. All strains were clinical isolates from humans and a variety of other animal species. These isolates represent the species S. typhi, S. choleraesuis, and S. enteritidis and a large number of serovars belonging to a variety of O-antigenic types.

Invasion by Salmonella strains of cultured Henle-407 cells was carried out in 24-well tissue culture plates as described (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). For qualitative screening of the invasiveness of a large number of Salmonella strains, a variation of this assay was performed essentially as described by Miller et al. (Miller, V.L., et al., Infect Immun (1989) 57:121-131). In brief, after gentamicin treatment, tissue culture cells were washed twice with Hanks balanced salt solution and lysed with 0.5 ml of the same solution containing 0.1% sodium deoxycholate. After 5 min, 1.5 ml of buffered saline containing 0.1% (wt/vol) gelatin was added to each well and 100 µl of the suspension was plated. Invasive strains gave almost confluent growth on the plates after overnight incubation, while noninvasive strains gave few isolated colonies.

All Salmonella strains tested, with the exception of strains of S. arizonae, were unambiguously shown to be invasive. Thirty strains were tested quantitatively, and the invasion values ranged from 1 to 30% (data not shown). These values represent the percentage of the initial inoculum that was insensitive to 2 h of gentamicin treatment because of invasion of tissue culture cells.

Example 2

Cloning the Invasion Genes from S. typhimurium

The invABC genes were originally cloned from S. typhimurium SR-11 as described (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). Specifically, a library of S. typhimurium SR-11 DNA was constructed in the cosmid vector pREG153 (Hull, R.A., et al., Infect Immun (1981) 33:933-938) following standard procedures (Maniatis, T., et al., Molecular Cloning: A Laboratory Manual (1982) (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.) and in vitro packaged into λ phage particles using a commercial packaging extract (Promega Biotec). The library was transduced into χ2819, in vivo packed as described (Jacobs, W.R., et al., Infect Immun (1986) 52:101-109), and stored as a lysate over chloroform at 4°C. The transfer of DNA to nylon membranes (GeneScreenPlus, DuPont) was carried out according to the method of Southern (J Mol Biol (1975) 98:503-517). Hybridizations were done with [^{32}P]dATP-labeled probes according to standard procedures (Maniatis T., et al., Molecular Cloning: A Laboratory Manual (1982) (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.). Phage P22 HT int transduction was performed as described (Davis, R.W., et al., Advanced Bacterial Genetics. A Manual for Genetic Engineering (1980) (Cold Spring Harbor Lab., Cold Spring Harbor, N.Y.)). Transformation of linear DNA was performed as indicated (Winans, S.C., et al., J Bacteriol (1985) 161:1219-1221). TnphoA mutagenesis was performed as described (Manoil & Beckwith, Proc Natl Acad Sci USA (1985) 82:8129-8133; Gutierrez, C., et al., J Mol Biol (1987) 195:289-297) utilizing λ O_{am} P_{am} rex::TNphoA cI857 b221 as the suicide transposon vector. In vitro transcription/translation of plasmid DNA was carried out using a kit from Amersham

according to the recommendations of the manufacturer. ^{35}S-radiolabeled proteins were separated on 10% polyacrylamide gels as described by Laemmli (Nature (1970) 227:680-685) and resolved polypeptides vizualized by fluorography with EN^{3}HANCE (DuPont).

In the process of screening strains of S typhimurium for their ability to invade cultured mammalian cells, it was observed that strain DB4673, although unable to penetrate Henle-407 cells, was able to adhere to these cells at levels equivalent to those of a wild-type strain. Strain DB4673 was, therefore, used as a recipient for a S. typhimurium SR-11 DNA library in the cosmid vector pREG153, to isolate genes that rendered DB4673 capable of invading monolayers of Henle-407 cells. A cosmid clone (pYA2217) was isolated which conferred on DB4673 the ability to penetrate Henle-407 cells as efficiently as its wild-type progenitor. No significant differences were observed in the ability of DB4673 to adhere to Henle-407 cells in the presence or absence of pYA2217. In contrast to Yersinia inv (Isberg, R.R., et al., Cell (1987) 50:769-778) and ail (Miller & Falkow, Infect Immun (1988) 56:1242-1248) genes, introduction of pYA2217 into E. coli strain HB101 did not confer upon this strain the ability to attach to or penetrate into Henle-407 cells. pYA2217 did not complement any of the known mutations in DB4673 or alter lipopolysaccharide structure as determined by SDS/polyacrylamide analysis and silver staining of lipopolysaccharide preparations.

Subcloning of the invasive phenotype in the plasmid vector pACYC184 yielded pYA2219 (Figure 2B). This plasmid was subjected to restriction endonuclease analysis and TnphoA mutagenesis to establish more precisely the regions conferring the invasive properties. Productive and nonproductive insertions of TNphoA into pYA2219 were mapped by restriction endonuclease analysis

and tested for their ability to penetrate Henle-407 cells. (TnphoA insertions are depicted in Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). Insertions which completely abolished the invasive phenotype mapped to a region of ~3.5 kilobases (kb) between the SalI and EcoRI sites at the left end of the insert. Another group of insertions that reproducibly diminished the invasive phenotype by 5-fold mapped to a 1-kb region between the two EcoRI sites at the right end of the 18-kb insert. The locus was designated inv to identify the different TnphoA insertion alleles. Insertion inv-61 and three other similar insertions (e.g., inv-20, inv-65, and inv-66) that completely abolished the invasive phenotype, yielded productive fusions to alkaline phosphatase indicating that these insertions most likely resided within the structural gene of a secreted protein (Manoil & Beckwith, Proc Natl Acad Sci USA (1985) 82:8129-8133). The direction of transcription of phoA in these insertions was from left to right. No other insertion generated a productive fusion.

Plasmid-encoded polypeptides were analyzed in a DNA-directed in vitro transcription/translation system. The pYA2219 insert DNA encoded at least six proteins (in addition to those encoded by the vector pACYC184) with the following molecular masses: 86, 64 (sometimes migrating as a doublet), 54, 47, 33, and 30 kDa were specified by TnphoA and both corresponded to similar size proteins encoded by pYA2219. Proteins encoded by a pYA2219inv-61::TnphoA insert generated a productive alkaline phosphatase fusion, inactivated the expression of the 64-, 54-, and 47-kDA polypeptides and generated a new protein of 50 kDa, a product of the fusion of alkaline phosphatase to the 54-kDa protein encoded by pYA2219. Insertion inv-11::TnphoA inhibited the

expression of the 64- and 47-kDa proteins. Both insertions inv-61::TnphoA and inv-19::TnphoA totally abolished the invasive phenotype of cells harboring pYA2219. Insertion inv-11::TnphoA, which diminished the invasion phenotype of cells with pYA2219 5-fold, blocked expression of the 30-kDa polypeptide. Insertions x67::TnphoA, x68::TnphoA, and x21::TnphoA did not affect the expression of any proteins encoded by pYA2219 and did not affect the invasive phenotype.

pACYC184::TnphoA encoded 54- and 33-kDa proteins that comigrated with two proteins encoded by pYA2219, preventing the unambiguous interpretation of the protein data. To address this issue, a series of overlapping deletions of pYA2219 were constructed by subcloning sequences from pYA2219 into pUC18 and were subjected to in vitro transcription/translation analysis. pYA2220 (Figure 2A) contains the leftmost end of the 18-kb insert DNA of pYA2219 from the HindIII to the closest PstI site and encodes three proteins of 64, 54, and 47 kDA in addition to those of pUC18.

Based on the in vitro transcription/translation analyses of pYA2219 insertion and deletion mutants, four genes were found to be involved in the invasive phenotype of DB4763 (pYA2219): invA, invB, invC, and invD, encoding proteins of 54, 64, 47, and 30 kDa, respectively. invA, invB, and invC are in the same transcriptional unit (Figure 2).

Example 3

Conservation of the invABC Operon in Salmonella Species and Serovars

To determine whether the invABC genes cloned in Example 2 were present in other Salmonella species and serovars and thus would be useful as probes for Salmonella, colony blot hybridization analysis was

performed on 91 Salmonella strains (Table 2). The probe used was a 3.4-kb ClaI-EcoRI fragment of pYA2220 (probe 1; Figure 2A) that contains most of the invABC operon, with no flanking sequences, as determined by preliminary sequence analysis. All Salmonella strains tested hybridized to the probe under high-stringency conditions (see Materials and Methods). No qualitative difference between the intensities of the signals of the positive control strain (S. typhimurium SR-11) and the other Salmonella strains was detected. The results indicate that these genes are not only present in all or most salmonellae but are also highly conserved.

To test the degree of restriction fragment length polymorphisms of the invABC genes, Southern blot hybridization analysis of a number of Salmonella strains was carried out. Strains tested included S. typhimurium (SR-11), S. dublin, S. enteritidis, S. choleraesuis, S. typhi, S. pullorum, S. gallinarum, S. arizonae, S. heidelberg, S. manhattan, S. newport, S. ohio, S. tennessee, S. duisburg, S. typhimurium, S. london, S. java, S. bovismorbificans, S. infantis, S. hadar, S. othmarschen, S. virchow, S. vejle, S. thompson, S. schwarzengrund, S. panama, and S. typhimurium (SR-11). Total-cell DNA samples were digested with EcoRI and PvuII and fragments were separated by electrophoresis through a 0.7% agarose gel and transferred to nylon membranes as described in Materials and Methods. Membranes were hybridized to a 4.5-kb SalI-PstI fragment of pYA2220 (probe 2; Figure 2A) that contains the entire invABC operon. This combination of probe and restriction enzymes gave information on polymorphisms of the invABC genes themselves and their flanking sequences, since EcoRI and PvuI generate internal fragments and probe 2 can hybridize to DNA fragments that span beyond the invABC operon. The 2.6-kb EcoRI-PvuII and 1.1-kb PvuII-

PvuII fragments internal to the invABC operon were conserved in all strains tested, except for S. arizonae strains, which showed a single high-molecular-weight hybridizing fragment. Some polymorphisms were observed in the restriction fragment containing sequences flanking the invABC genes.

Example 4

Conservation of invD in Salmonella Strains

As described above, the invD locus was originally identified by transposon insertional mutagenesis of pYA2219, a plasmid that contains DNA from S. typhimurium SR-11 and was able to complement an invasion-deficient strain of S. typhimurium (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387). Transposon insertions in the invD locus diminished, but did not abolish, the complementing ability of pYA2219 and also eliminated the expression of a 30-kDa polypeptide in an in vitro transcription-translation system (Galan & Curtiss, Proc Natl Acad Sci USA (1989) 86:6383-6387).

To test for the presence of invD-like sequences in other Salmonella strains, high-stringency colony blot hybridization analysis was performed as described above. The probe used was a 2.4-kb EcoRI fragment of pYA2219 that contains the invD locus (probe 3; Figure 2B). The precise boundaries of this gene have not yet been determined, but it is expected that probe 3 has sequences that flank invD, since some transposon insertions in pYA2219 that mapped near one end of the 2.4-kb EcoRI fragment did not affect the complementing ability of this plasmid and therefore are assumed to be outside invD. In addition, probe 3 contains more DNA than would be needed to encode a 30-kDa polypeptide, the product of invD.

All Salmonella strains tested (Table 2) hybridized to the probe, although the two S. arizonae strains tested

showed a weaker signal than did the positive control strain (S. typhimurium SR-11). These data suggested that the invD gene may be present in most (if not all) Salmonella strains tested.

Southern blot hybridization analysis was used to study the conservation among Salmonella strains of the 2.4-kb EcoRI fragment that contains the invD region, a better indicator of the distribution of the invD locus. The same blots as those used in the invABC analysis were utilized, since there are no PvuII sites within the 2.4-kb EcoRI fragment that contains the invD region. The 2.4-kb fragment was present in most of the strains tested, although several strains showed additional hybridizing fragments. The latter may indicate the existence of more than one copy of this gene in some strains. Three strains (isolates of S. choleraesuis, S. typhi, and S. panama) showed a different pattern of hybridization. An isolate of S. arizonae that had shown a weak signal in the colony blot hybridization analysis showed a weak high-molecular-weight hybridizing fragment upon prolonged exposure. These results strongly suggest that the invD locus is widely distributed among most Salmonella species and serovars and therefore useful as an agent to detect the same.

Example 5

Construction of invA Mutants of Different Salmonella Species and Serovars

Having established the presence of invABC-homologous sequences in all Salmonella strains analyzed, it was of interest to see whether these genes were functional. Therefore, invA mutants (unable to express the invABC genes) of S. typhi, S. gallinarum, S. dublin, and S. enteritidis were constructed by transducing these strains to kanamycin resistance with a P22HTint lysate

prepared on strain SB103. SB103 is an S. typhimurium strain that carries an insertion of a kanamycin resistance gene cassette in the ClaI site of invA. The correct position of invA::kan in the transductants was verified by Southern blot hybridization analysis. invA mutants were tested for their ability to penetrate cultured Henle-407 cells. The results of these experiments are shown in Table 3. invA mutants of S. typhi (SB129 and SB130), S. enteritidis (SB131), S. gallinarum (SB132), and S. dublin (SB133) were significantly impaired in their ability to penetrate cultured epithelial cells. These data indicate that, in these strains, the invABC genes are not only present but also functional.

TABLE 3. Invasion by Salmonella strains of Henle-407 cells

Strain	Species or serovar	Relevant genotype	% Invasion[a]
SL1344	S. typhimurium	Wild type	19.2 ± 6.1
SB103	S. typhimurium	invA::kan	0.02 ± 0.008
Ty2	S. typhi	Wild type	7.46 ± 5.2
SB129	S. typhi	invA::kan	0.07 ± 0.04
2825	S. typhi	Wild type	25.2 ± 1.9
SB130	S. typhi	invA::kan	0.03 ± 0.003
7193	S. enteritidis	Wild type	12.9 ± 2.15
SB131	S. enteritidis	invA::kan	0.04 ± 0.006
Stock	S. gallinarum	Wild type	30.6 ± 5.6
SB132	S. gallinarum	invA::kan	0.1 ± 0.02
Lane	S. dublin	Wild type	27.6 ± 2.4
SB133	S. dublin	invA::kan	0.258 ± 0.054
77-85	S. arizonae	Wild type	0.515 ± 0.01
875-84	S. arizonae	Wild type	0.07 ± 0.01

[a] Invasion is expressed as the percentage of the initial inoculum of bacteria that was insensitive to gentamicin because of cell invasion. The values represent the averages ± standard deviations for three samples. Similar results were obtained in several repetitions of this experiment.

Example 6

Conservation of invA in Salmonella Strains

The invA gene isolated above, was sequenced using standard techniques. The sequence is depicted in Figure 1, beginning at nucleotide position 1474. Based on this sequence, primers were synthesized using standard techniques. See, Oligonucleotide Synthesis, Gait, M.J., ed. (IRL Press), 1984. The primers consisted of the following nucleotide sequences:

(5'-3') GTGAAATTATCGCCACGTTCGGGCAA; and

(5'-3') TCATCGCACCGTCAAAGGAACC.

These primers were used in PCR amplification studies of 636 strains of Salmonella belonging to over 100 serotypes. PCR was carried out using standard techniques, well known in the art, using a Hybaid thermal reactor. Taq polymerase was used. See, e.g. U.S. Patent No. 4,683,195 and 4,683,202 and Saiki, R.K., et al., Science (1988) 239:487-491. Of the 636 strains tested, 634 (99.4%) were specifically detected. In addition, as described below, no non-Salmonella strains were specifically amplified.

Example 7

Absence of the inv Genes in Other Invasive Enteric Bacteria

Several enteric bacteria other than Salmonella strains have been shown to invade cultured epithelial cells (Miller, V.L., et al., Curr Top Microbiol Immunol (1988) 138:15-39; Small, H., Infect Immun (1987) 55:1674-1679). Accordingly, several of these strains, including Yersinia spp., Shigella spp., and enteroinvasive and enteropathogenic E. coli, were tested for DNA sequences similar to invABC or invD. The 3.4-kb ClaI-EcoRI fragment of pYA2220 (probe 1; Figure 2A) that contains

most of the invABC operon with no flanking sequences was used to detect invABC-like sequences, and the 2.4-kb EcoRI fragment of pYA2219 that contains the invD locus (probe 3, Figure 2B) was used to detect invD-like sequences. Colony blot hybridization analysis was performed under low-stringency hybridization conditions (see Materials and Methods). Under these conditions, neither of the probes hybridized to any of the strains tested.

Similarly, several organisms were tested with the invA primers described above. None of these strains were specifically amplified using these primers.

These results indicate that inv-like sequences are absent in related organisms and thus these sequences are specific for Salmonella.

Thus, methods for detecting the presence of Salmonella in biological samples are disclosed as well as probes and primers for use in the same. Although preferred embodiments of the subject invention have been described in some detail, it is understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

Claims

1. An oligomer that is a recombinant polynucleotide capable of hybridizing to a Salmonella nucleotide sequence in an analyte strand, said oligomer comprising at least 8 contiguous nucleotides derived from a Salmonella typhimurium inv gene.

2. The oligomer of claim 1 wherein said at least 8 contiguous nucleotides are derived from the Salmonella typhimurium invA gene or the InvB gene or the Inv C gene or the invD gene.

3. The oligomer of claim 2 wherein said at least 8 contiguous nucleotides are derived from the sequence GTGAAATTATCGCCACGTTCGGGCAA.

4. The oligomer of claim 2 which comprises the nucleotide sequence GTGAAATTATCGCCACGTTCGGGCAA.

5. The oligomer of claim 2 wherein said at least 8 contiguous nucleotides are derived from the sequence TCATCGCACCGTCAAAGGAACC.

6. The oligomer of claim 2 which comprises the nucleotide sequence TCATCGCACCGTCAAAGGAACC.

7. The oligomer of claim 2 which comprises probe 3 of figure 2B.

8. The oligomer of claim 1 which comprises a polynucleotide sequence derived from the Salmonella typhimurium invABC operon, said nucleotide sequence comprising at least a portion of the Salmonella typhimurium invA gene, at least a portion of the

Salmonella typhimurium invB gene, and at least a portion of the Salmonella typhimurium invC gene.

9. The oligomer of claim 8 which comprises probe 1 or probe 2 of figure 2A.

10. The oligomer of claim 1 which is a primer.

11. A method for detecting the presence or absence of a Salmonella polynucleotide sequence in an analyte strand, which method comprises:

(a) providing at least one oligomer capable of hybridizing to a Salmonella target sequence in a target region of an analyte strand, said at least one oligomer comprising a Salmonella targeting sequence complementary to at least 8 contiguous nucleotides derived from a Salmonella typhimurium inv gene; and

(b) incubating said analyte strand with said at least one oligomer under conditions which allow specific hybrid duplexes to form between said Salmonella target sequence and said Salmonella targeting sequence,

thereby detecting the presence or absence of said Salmonella polynucleotide sequence.

12. The method of claim 11 wherein said at least 8 contiguous nucleotides are derived from the Salmonella typhimurium invA gene or the invB gene or the invC gene or the invD gene.

13. The method of claim 12 wherein at least one oligomer comprises probe 3 of figure 2B.

14. The method of claim 11 wherein at least one oligomer comprises a polynucleotide sequence derived from the Salmonella typhimurium invABC operon, said

polynucleotide sequence comprising at least a portion of the Salmonella typhimurium invA gene, at least a portion of the Salmonella typhimurium invB gene, and at least a portion of the Salmonella typhimurium invC gene.

15. The method of claim 21 wherein at least one oligomer comprises probe 1 or probe 2 of figure 2A.

16. The method of claim 11 wherein at least one oligomer comprises a set of oligomers which are primers for the polymerase chain reaction method and which flank the target region, and wherein said method further comprises amplifying said target region via the polymerase chain reaction.

17. The method of claim 16 wherein said primers are derived from the Salmonella typhimurium invA gene.

18. The method of claim 17 wherein one of said primers comprises the nucleotide sequence GTGAAATTATCGCCACGTTCGGGCAA and the other of said primers comprises the nucleotide sequence TCATCGCACCGTCAAAGGAACC.

19. A kit for detecting the presence or absence of a Salmonella polynucleotide sequence in an analyte strand, said kit comprising the oligomer of claim 1 packaged in a suitable container.

DNA sequence 5321 b.p. AATTGGATTAGC ... TCAGGATGAGGA linear

Positions of Restriction Endonucleases sites (unique sites underlined)

FIG. IA

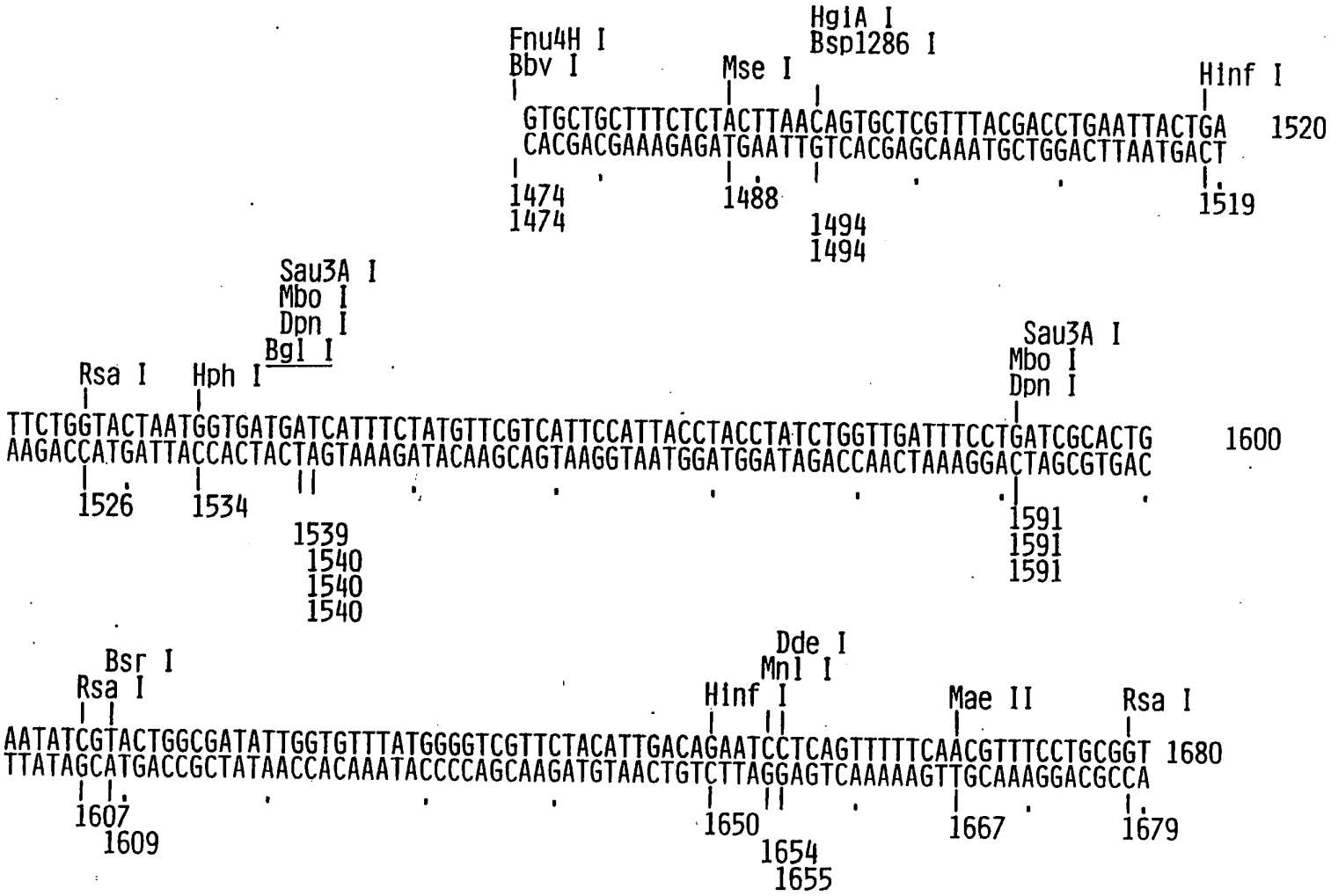
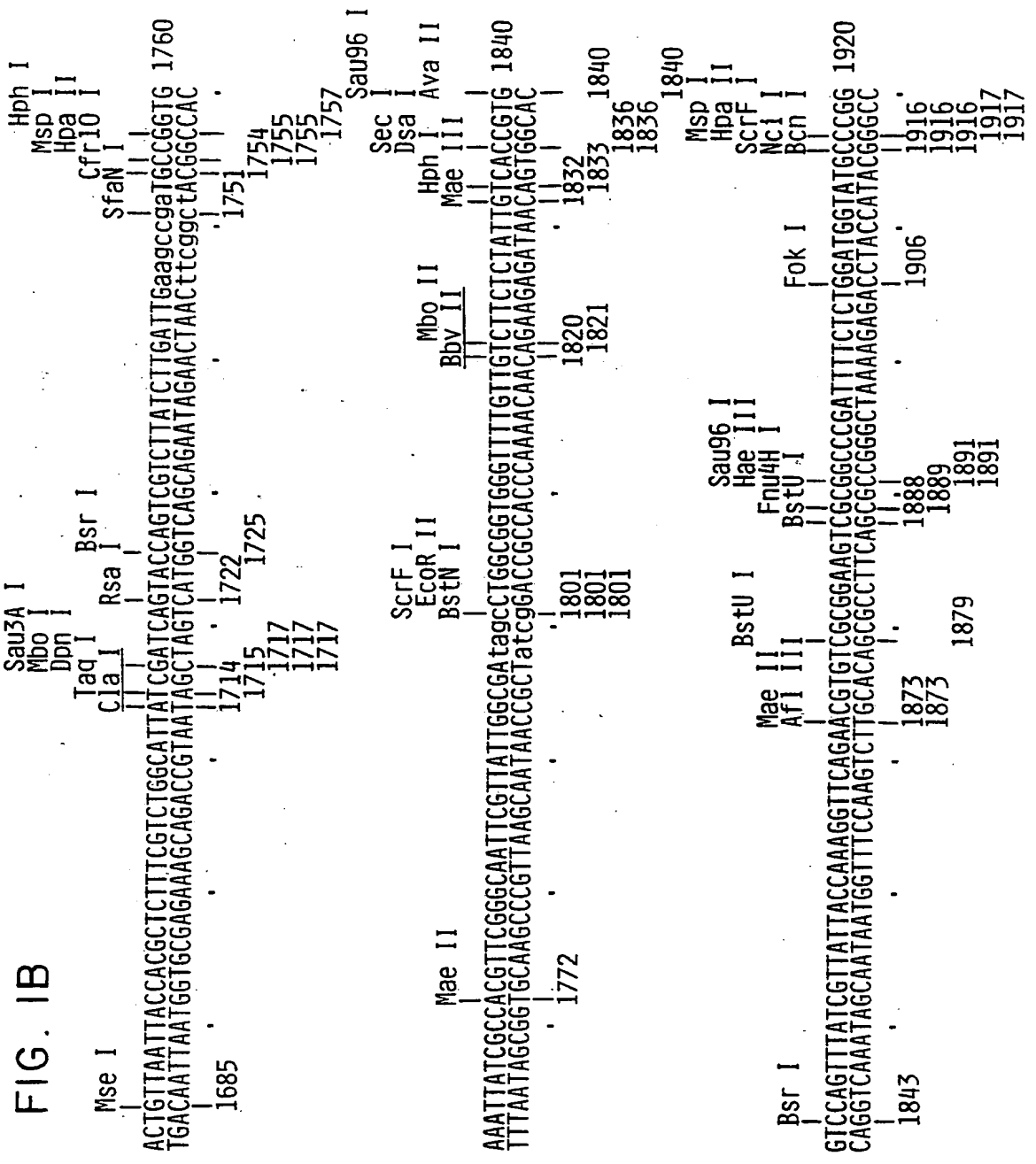


FIG. 1B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1757
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Sec I
Dsa I
Hph I
Ava II
Mae III
Mbo II
Bdv II
ScrF I
EcoR II
BstN I
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Mae II
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1801
1801
1801
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1821
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1840
Msp I
Hpa II
ScrF I
Nci I
Bcn I
Sau96 I
Hae III
Fnu4H I
BstU I
Mae II
Afl III
Bsr I
Fok I
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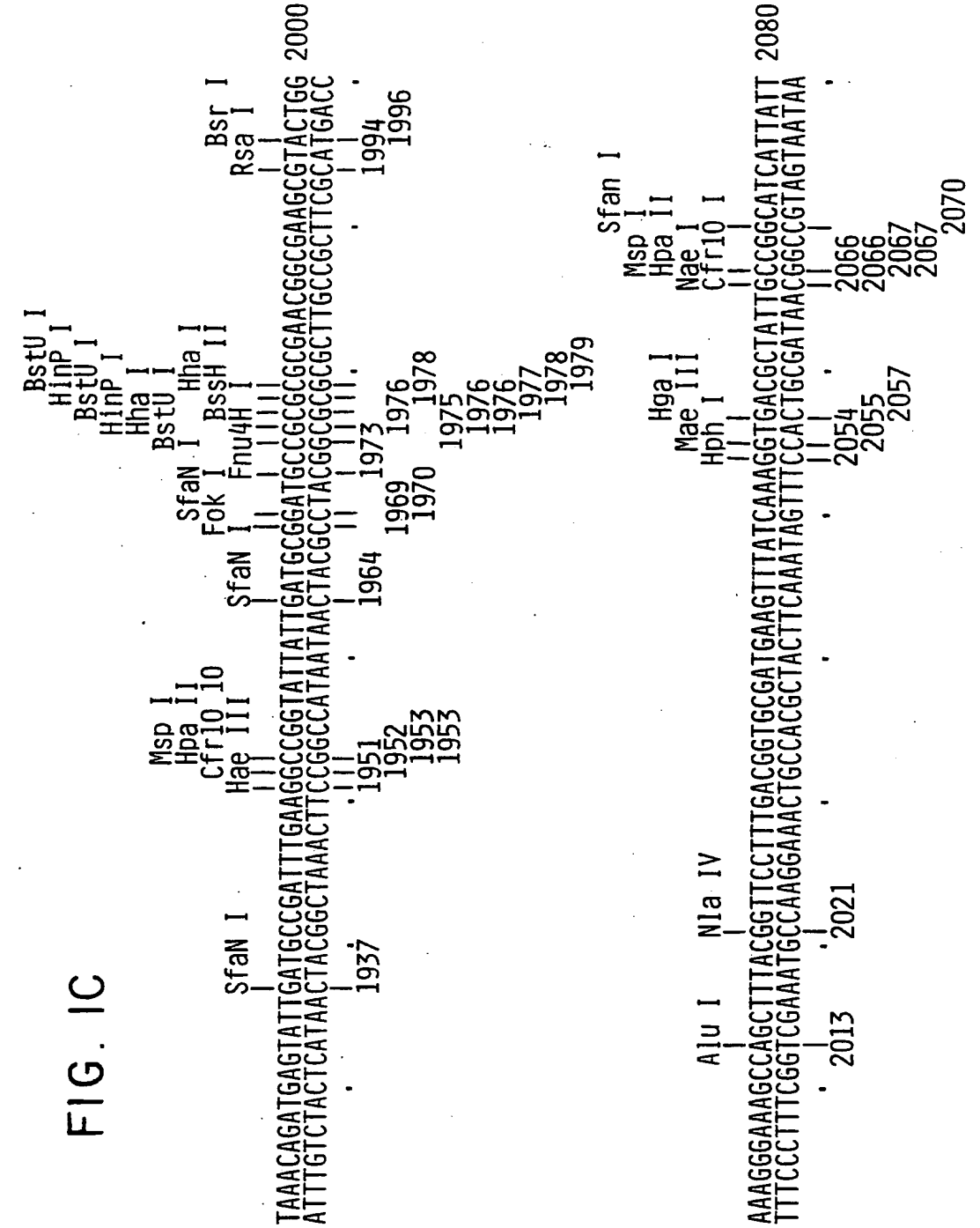
FIG. IC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SfaN I
Fok I
Fnu4H I
BssH II
Bsr I
Rsa I
Sfan I
Msp I
Hpa II
Nae I
Cfr10 I
Hga I
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Hph I
Alu I
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1951
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1964
1969
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1976
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1978
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1996
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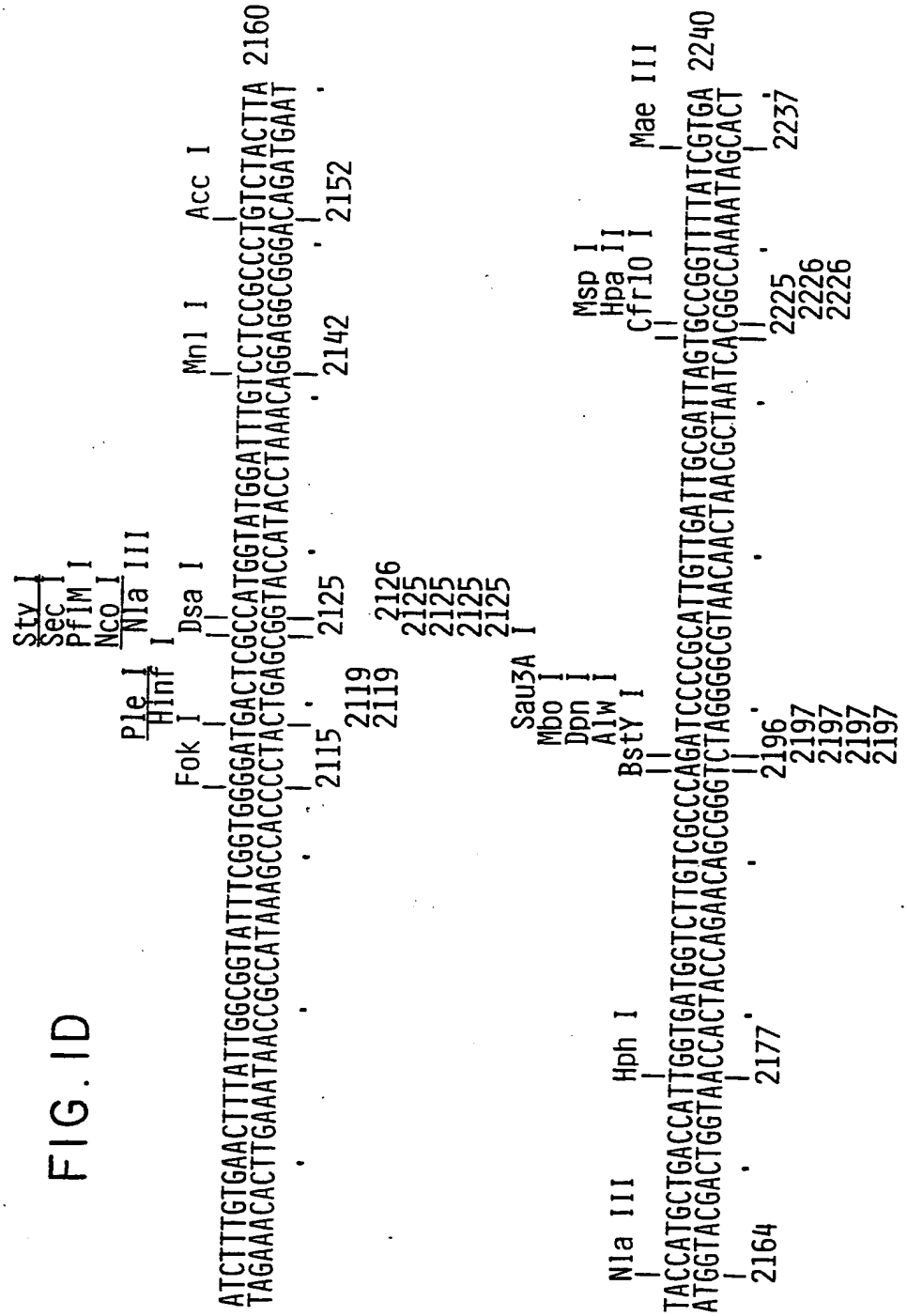
FIG. 1D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Cfr10 I
Mae III
Sau3A I
Mbo I
Dpn I
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BstY I
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2152
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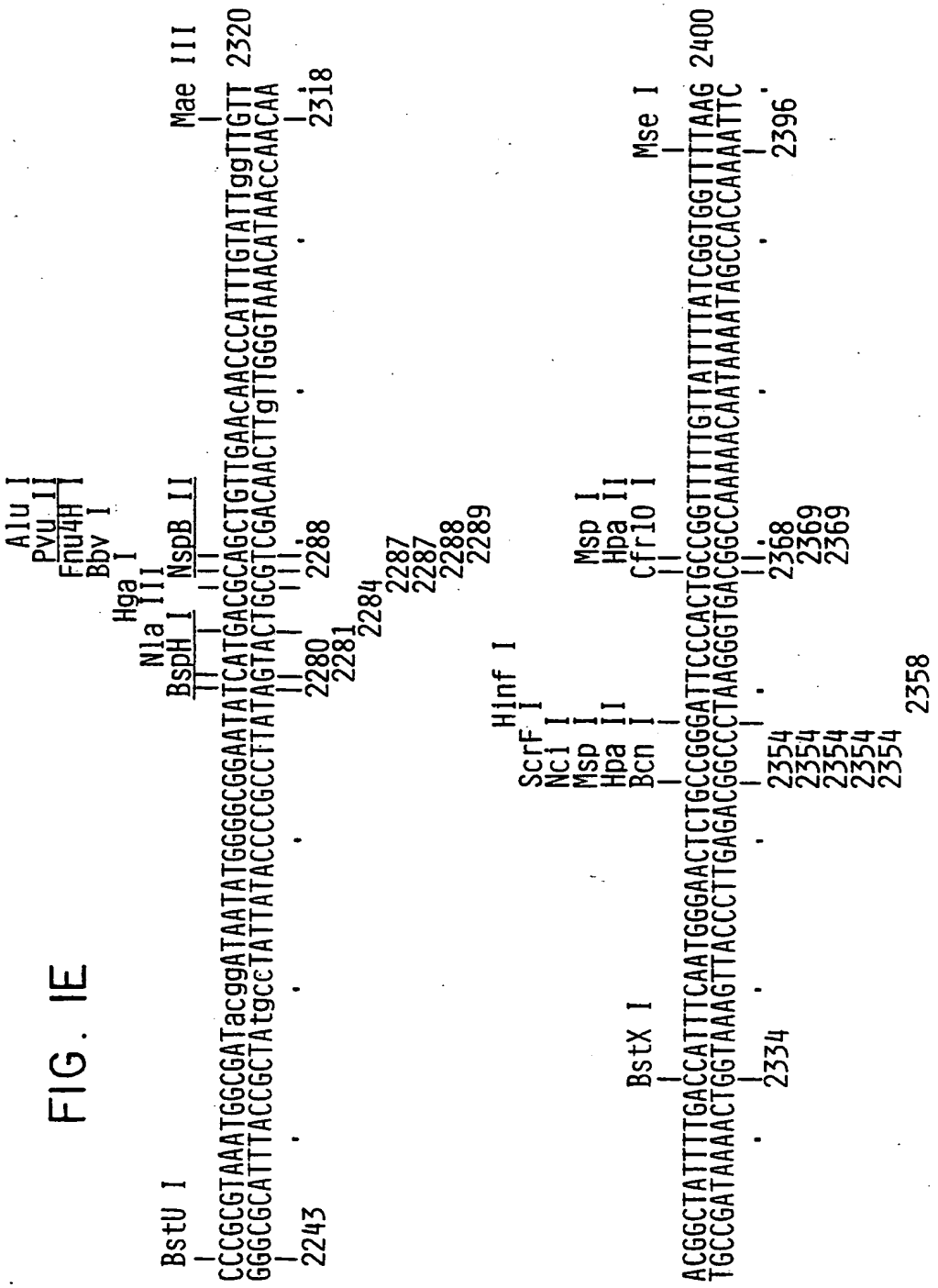
FIG. 1E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2288
2289
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2318
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Nci I
Msp I
Hpa I
Bcn I
Msp I
Hpa II
Cfr10 I
Mse I
BstX I
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2400

FIG. 1F

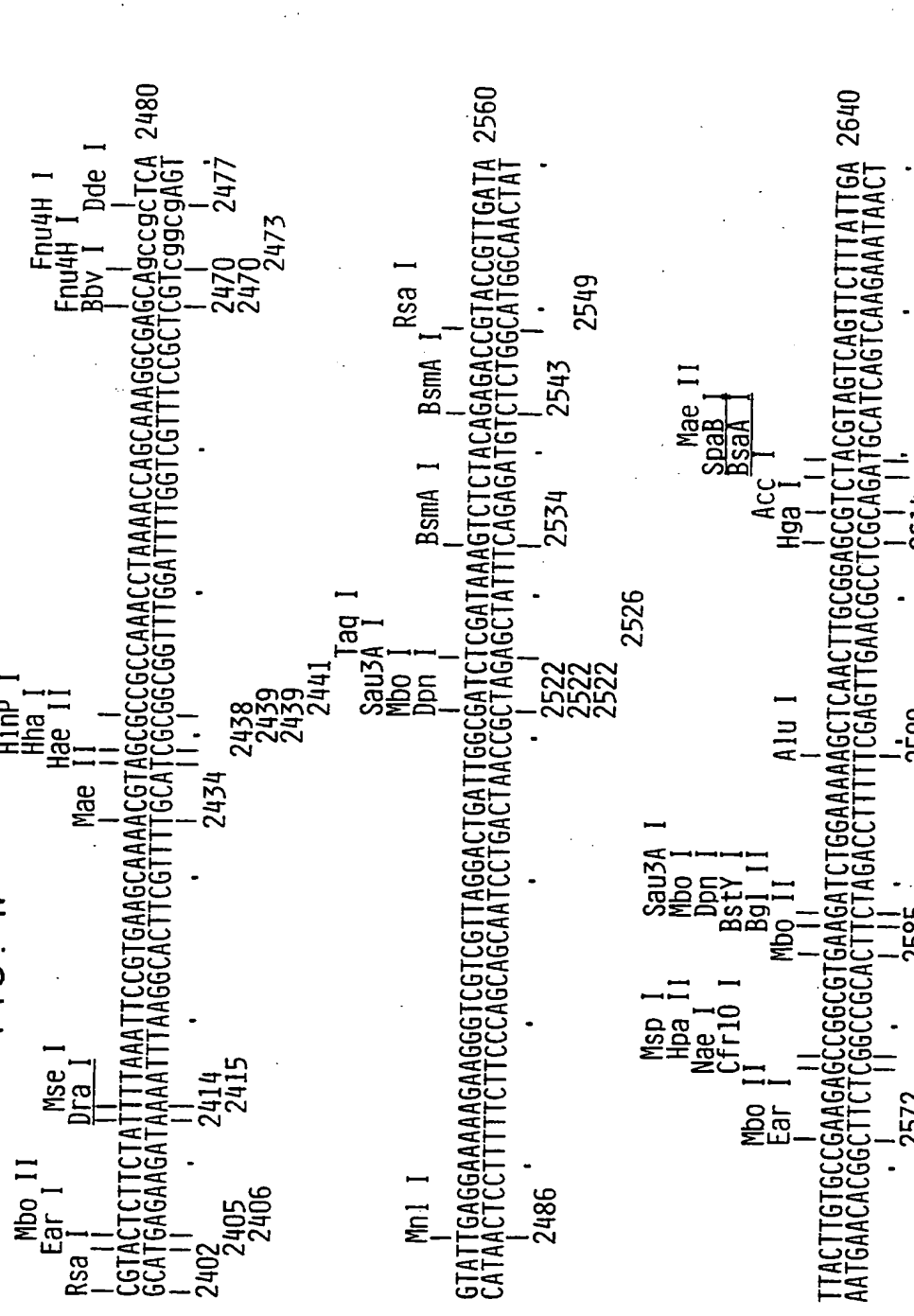
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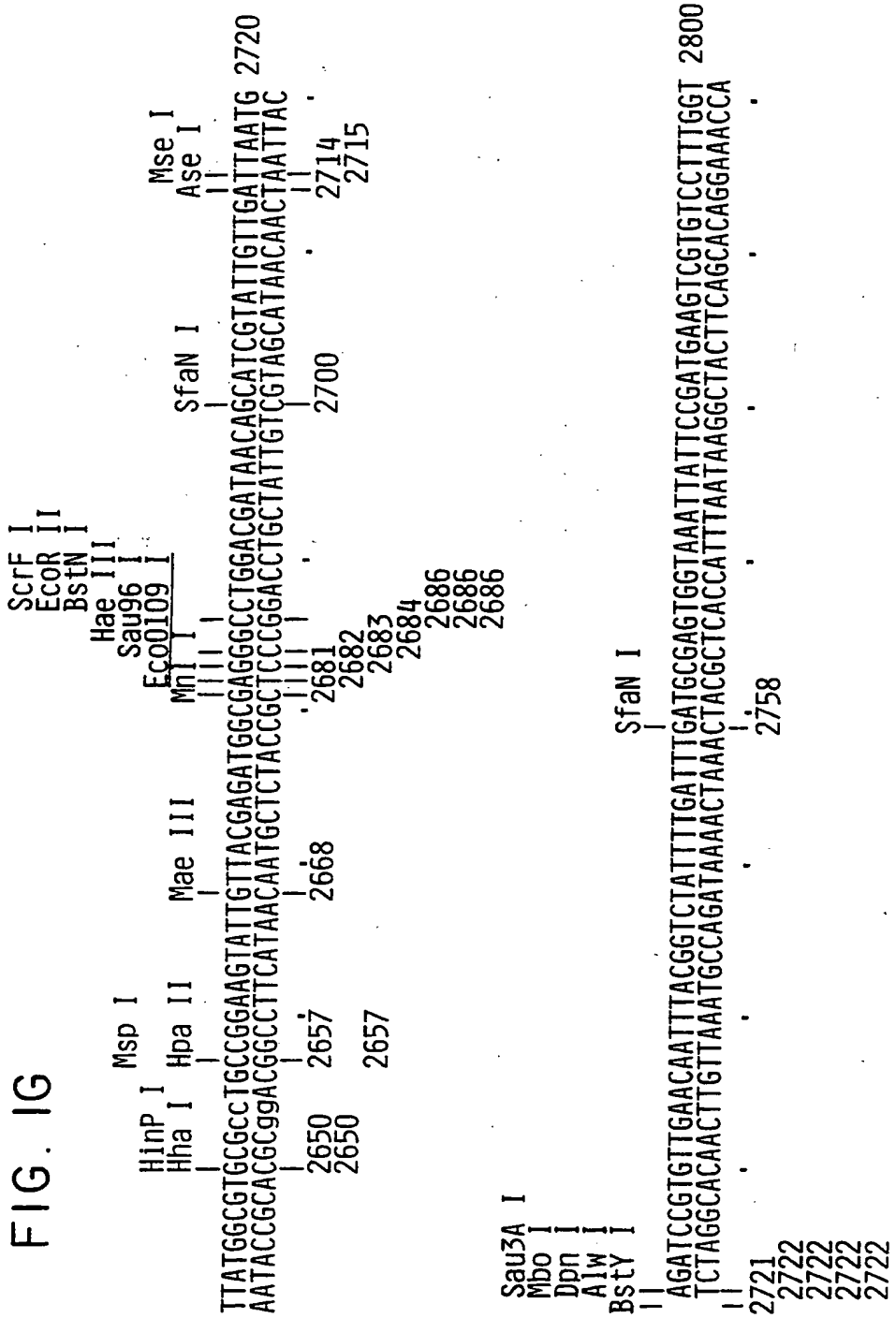
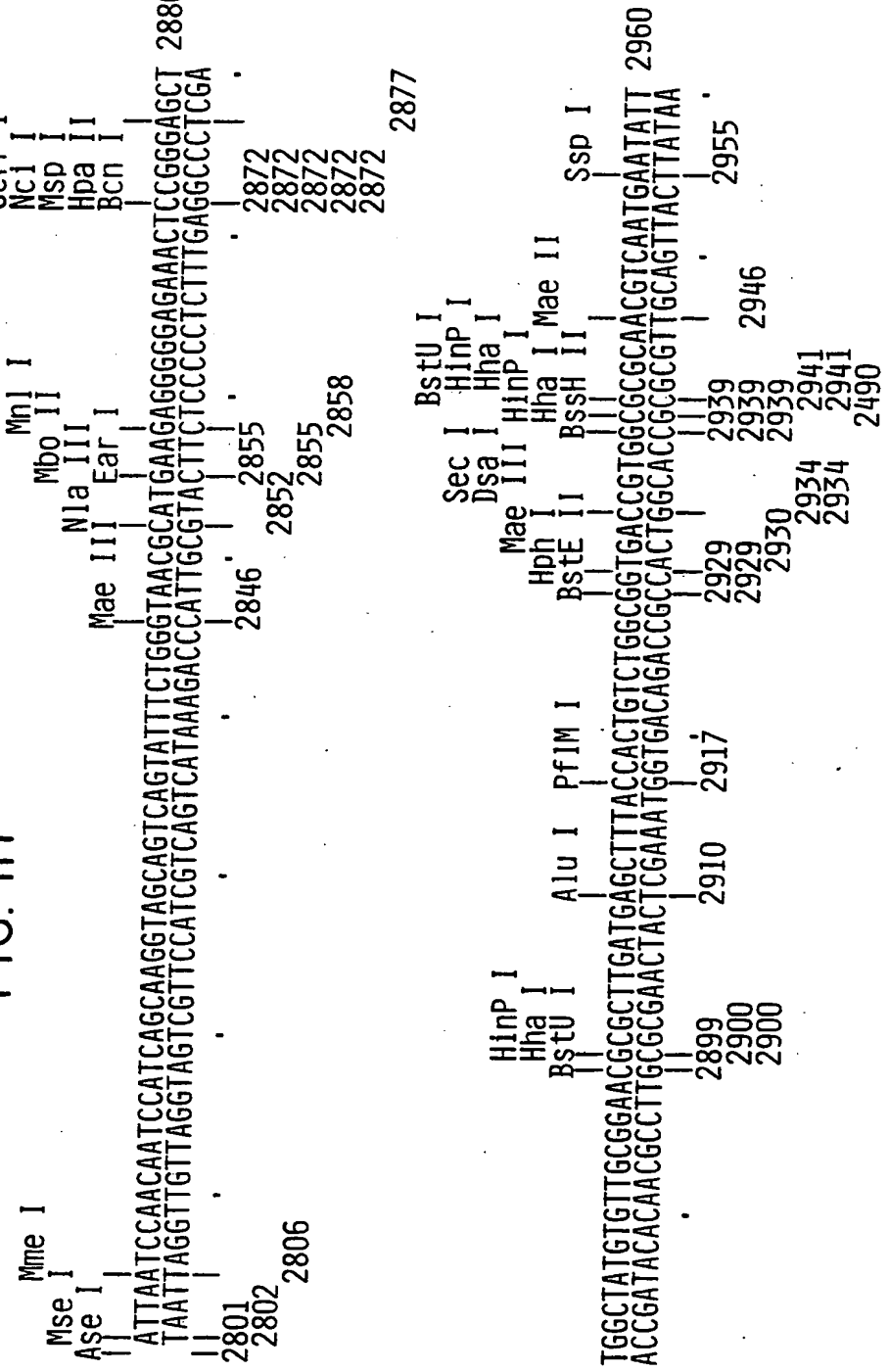
FIG. 1G



SUBSTITUTE SHEET

FIG. 1H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Bcn I
Alu I
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Hha I
BstU I
Alu I
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FIG. 11

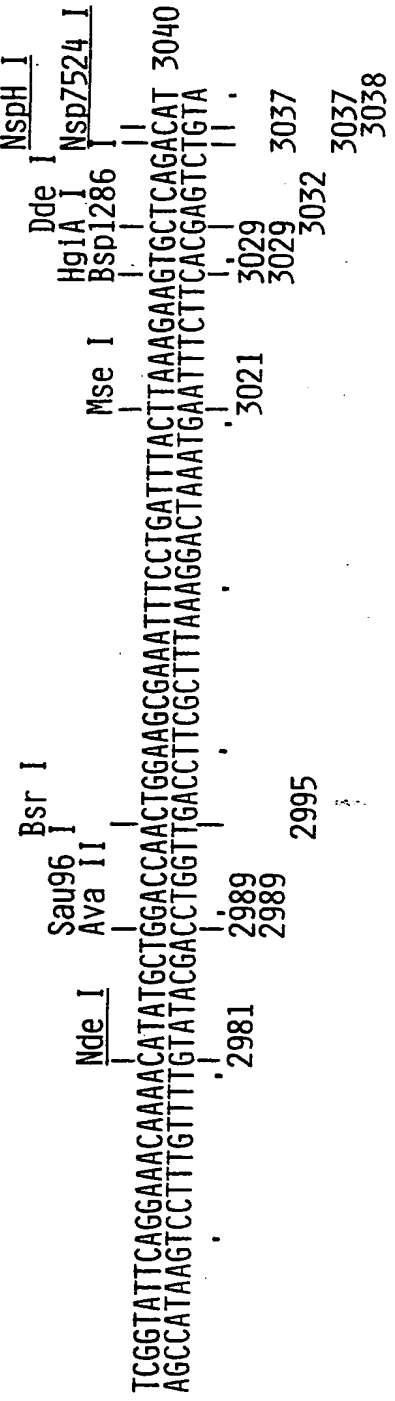
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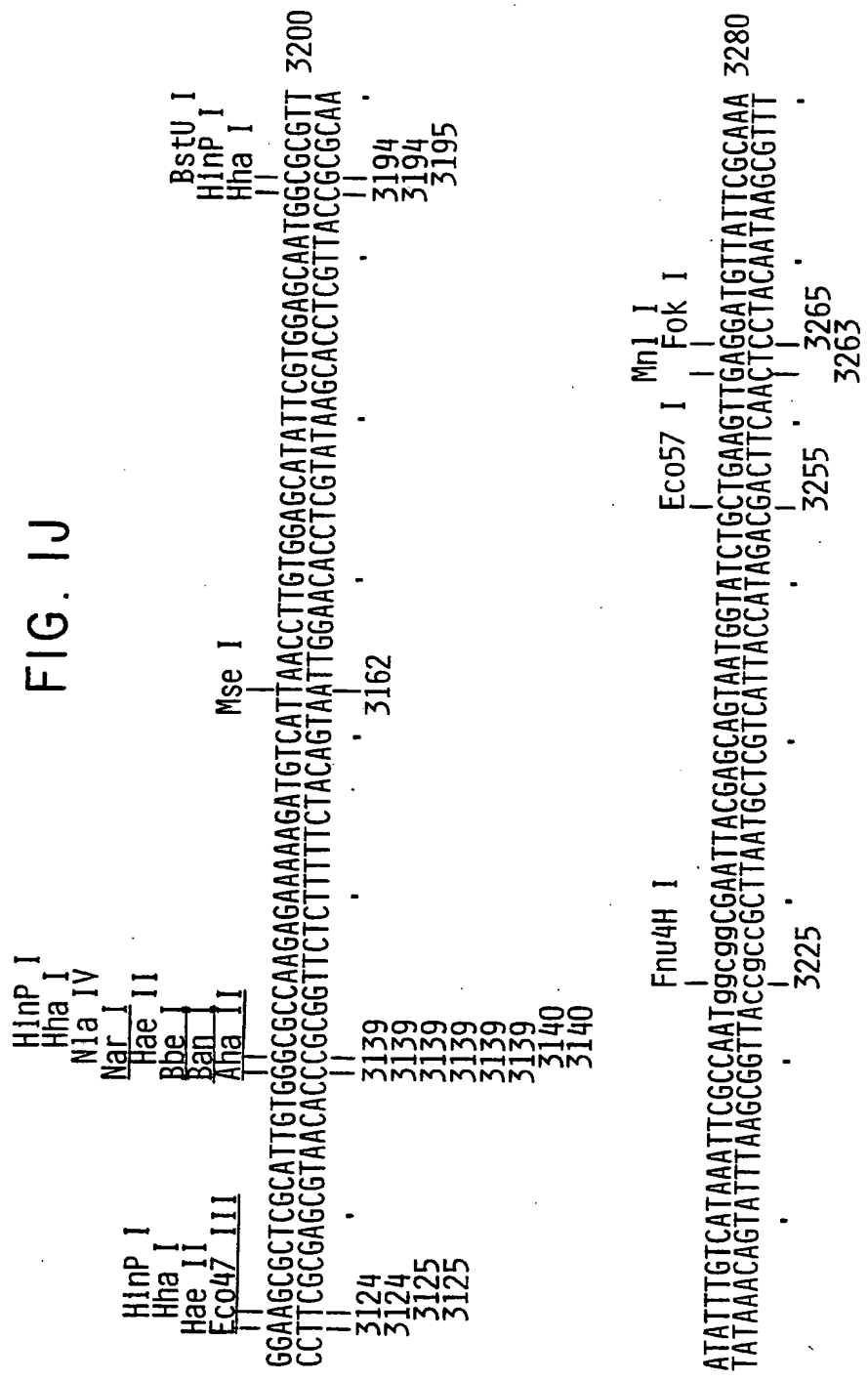
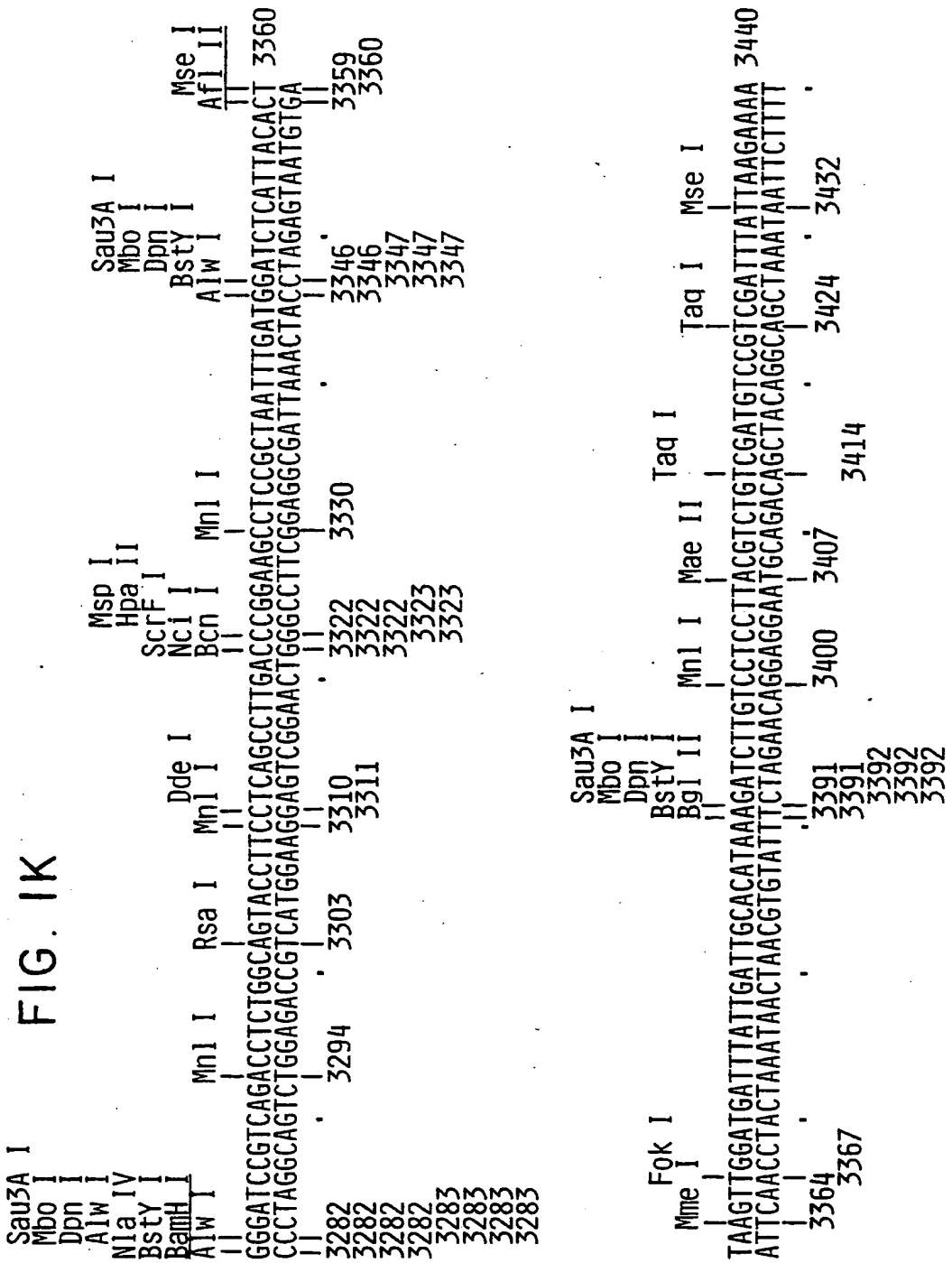
FIG. IJ



SUBSTITUTE SHEET

FIG. 1K



SUBSTITUTE SHEET

FIG. 1L

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Dpn I
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AIw I
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BspM II
Mnl I
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Acc I 2 9
Afl II 1 1
Afl III 2 7
Aha II 1 -
Alu I 5 6
AIw I 6 1
AlwN I - 4
Apa I - 11
ApaL I - 1
Ase I 2 -
Asp718 - -
Ava I - 4
BstN I
BstU I
BstX I
BstY I
Bsu36 I
Cfr10 I
Cla I
Dde I
Dpn I
Dra I
Dra III
Drd I
Dsa I
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HinD III - -
HinF I 4 -
HinP I 10 -
Hpa I - -
Hpa II 12 -
Hph I 7 -
Kpn I - -
Mae I - -
Mae II 9 -
Mae III 7 -
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Mbo II 5 -
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Pml I - -
PpuM I - -
Pst I - -
Pvu I - -
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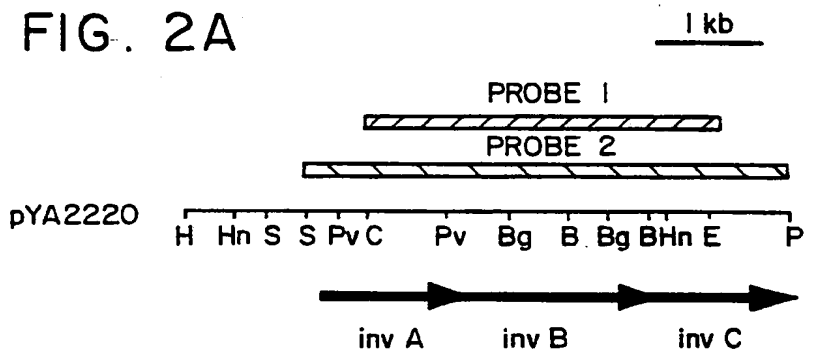
FIG. 2A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PROBE 2
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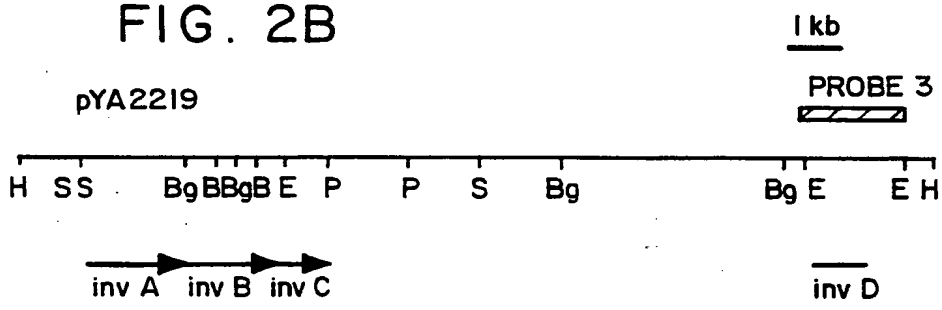
inv A
inv B
inv C

FIG. 2B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inv A
inv B
inv C
inv D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/06984

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C07K 3/10; C12P 19/34
US CL :435/6, 91; 536/27
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/27; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, World Patents, EMBASE, GENEBANK, EMBL
Search terms: Salmonella typhimurium and inv, S. typhimurium and PCR or hybridization

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings National Academy of Sciences, USA, Volume 86, issued August 1989, J. E. Galan et al, "Cloning and Molecular Characterization of Genes Whose Products Allow Salmonella typhimurium to Penetrate Tissue Culture Cells", pages 6383-6387, especially pages 6384 and 6385.	1-19
Y	EP, A2, 0,355,989 (Hiroyuki et al) 28 February 1990, page 2.	11-15, 19
Y	Bio/Technology, Volume 8, issued March 1990, T. Barry et al, "A General Method to Generate DNA Probes for Microorganisms", pages 233-236, especially pages 233 and 234.	16-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
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